# VALUE ADDITION AND NUTRIENT RECOVERY FROM ETHANOL COPRODUCTS

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## Abstract

Grain-to-ethanol production has increased steadily in the United States in the past few decades, which resulted in remarkable records in the availability of coproducts. Dry-grind is the most common method of ethanol production worldwide, which concentrates the corn and yeast nutrients in the downstream operations. The industrial corn ethanol process consists of a series of chemical, physical and biological operations. Once the fermented corn mash is produced, it is then processed through a series of distillation and rectification, centrifugation and evaporation processes to generate ethanol and the main co-product, Distillers Dried Grain with Solubles (DDGS). Through a series of condensers, the stillage is dried to produce DDGS. Thus, protein, fat, fiber, and phosphorus concentrations are increased several fold in the DDGS compared with corn. The remaining nutrients from the ethanol distillation are the raw material for producing coproducts. They are composed of proteins, fats, sugars, and some other lignocellulosic-derived compounds. In the United States, abundant research has been done in improving the quality of the ethanol coproducts which yielded the ethanol industry to have animal feed production to be as relevant as the fuel ethanol production. The liquid fraction produced after the centrifugation of the bottoms of the ethanol rectifying and distilling operations is named thin stillage, produced at volumes several times greater than those of ethanol. A portion of thin stillage is normally recycled as backset water, while the remaining goes through a series of evaporations. Evaporating a large amount of water from thin

stillage is an energy-consuming process and recycling the thin stillage may lead to the accumulation of nutrients in coproducts in distiller's grains. There are several other industrial processes to utilize thin stillage, such as oil extraction, anaerobic digestion, and secondary fermentation. Recently, promising results have been reported on the production of important commodity chemicals, extracting high-value products, and energy production from thin stillage.

Phytate is the primary storage form of phosphorus and inositol in plants. The bioavailability of phosphorus bound to phytate is low for non-ruminants, and thus the phytate-derived phosphate ingested by these species (e.g. poultry and swine) is largely excreted, resulting in both nutritional deficiencies and environmental pollution.

On the other hand, phytate is a widely applied valuable chemical as human nutrition, pharmaceutics, cosmetics, chelating chemical, and the raw material to produce myo-inositol. It is primarily produced from rice bran in Asia and imported to the US market. This creates a great opportunity for the US ethanol industry because extracting phytate as a new product from corn ethanol coproducts, knowing that corn germ has up to 5% phytate in its composition, can create additional revenue while increasing the feeding value of coproducts and decrease the phosphorus excretion in the animal manure.

This dissertation also provides an overview on the new processes and products via valorization of thin stillage by innovative technologies that are being currently developed. It also provides the technical development background to

develop an ion exchange method to extract phytate from thin stillage, as well as potential environmental benefits and further analyses on the impacts of phytate extraction implementation to ethanol plants. The novel application of thin stillage discussed in this dissertation could open new opportunities for the ethanol plants and ethanol researchers by increasing the revenue and simultaneously potentially reducing negative environmental impacts of ethanol production.

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# CHAPTER 1: INTRODUCTION

## 1.1. Dissertation structure

This dissertation is distributed across 8 chapters and two appendices. Eight of these are either published, in peer-review or in preparation form for submission. Chapter 2 is a literature review published on Reviews in Environmental Science and Bio/Technology summarizing the recent technologies applied to corn-to-ethanol thin stillage. Chapter 3 is an article to be submitted at Royal Society of Chemistry's Environmental Science: Processes and Impacts. Chapter 4 is published at Royal Society of Chemistry's RSC Advances, the same journal Chapter 6 will be submitted to. Chapter 5 is published at American Chemical Society's Industrial and Engineering Chemistry Research. Chapter 7 is in submission preparation to BioEnergy Research. Appendix 1 is in preparation for submission and Appendix 2 is published at Frontiers in Energy Research.

## 1.2. Background – Dry-grind ethanol production

Commercial production of fuel ethanol involves breaking down the starch present in corn to simple sugars, followed by the bioconversion of sugar molecules to ethanol catalyzed by yeast. The downstream processing involves the recovery of ethanol by distillation and the residual coproducts are further processed to serve as animal feed. Accounting for over 70% of the total ethanol production in the United State, dry-grind differs from the wet-milling process by the absence of an initial steeping operation to the corn grain. Most of the coproducts of interest are separated before the fermentation process in wet-milling industries. In the dry-grind process, the residual components are separated after the fermentation and

distillation.

The industrial corn ethanol process consists of a series of chemical, physical and biological operations, which are milling, liquefaction, saccharification, fermentation and distillation. Once the fermented corn mash is produced, it is then processed through a series of distillation, centrifugation and evaporation processes to generate ethanol and the main coproduct, Distillers Dried Grain with Solubles (DDGS).

The remaining nutrients from the ethanol distillation are the raw material for producing coproducts. They are composed of proteins, fats, sugars, and some other lignocellulosic-derived compounds. In the United States, abundant research has been done in improving the quality of the ethanol coproducts which yielded the ethanol industry to have animal feed production to be as important as the fuel ethanol production. Among the coproducts streams in the dry-grind ethanol industry are DDGS and other animal feed products, biogas produced through anaerobic digestion from stillage, and corn oil.

About two-thirds of corn is starch, which is fermented to ethanol in the dry-grind process. The remaining nutrients are recovered in the stillage. Through a series of condensers, the stillage is dried to produce DDGS. Thus, protein, fat, fiber, and phosphorus concentrations are increased several fold in the DDGS compared with corn.

When coproducts are fed as an energy source to animals (above 15 to 20% of diet), protein and phosphorus can be overfed. The environmental impact of

feeding protein and phosphorus-rich materials to animals is related to one of the most pervasive forms of pollution from modern agriculture, as animal manure is applied to the soil. As manure is applied as fertilizer, the excess non-bioavailable phosphorus compounds tend to accumulate in the soil and causing eventual runoffs to waterbodies, causing eutrophication and the formation of hypoxic zones.

Two major processes used to produce ethanol in the United States are the wet-milling and the dry-milling. The wet-milling process starts with a steeping operation on the corn grain, which separates it into starch, germ, and fibers (Wang, Wu, & Huo, 2007). In dry-milling process corn flour is produced through a grinding process, which is processed without separation of starch. This latter requires lower capital investment and presents an easier operation.

The corn-to-ethanol process on a dry-grind plant is summarized through the following operations from the upstream through the first downstream steps: milling, mashing, cooking, liquefaction, saccharification, fermentation, distillation, solids separation, evaporation, and $CO_2$ scrubbing (Reis et al. 2017). The milling step accounts for a hammering of corn, grinding it to a fine powder called meal. Corn meal is mixed with water, derived from corn grains, makeup water, recycled water, water condensate from evaporators, and $CO_2$ scrubber water, producing corn mesh. Alpha-amylase is added to the slurry mixer to start the degradation of starch to dextrin (Belyea et al., 2004). Corn slurry is then cooked using a hydroheater. Steam enters the cooking system to heat the slurry and a pressure

drop of up to 2.8 atm is applied to facilitate the mechanical shearing of starch molecules. Slurry is heated to 90-120 ºC, and it is held in this temperature for up to 10 minutes, with a saturation pressure of about 2 atm. The high temperature and pressure in this cooking step help reducing the bacteria levels in corn mash (Belyea et al., 2004).

The cooked mash is liquefied, partially converting the gelatinized starch to soluble dextrin, decreasing significantly the viscosity. Additional alpha-amylase is added to this step, since the cooking process denatures most of the previously-added enzymes. The yeast propagation process starts aerobically to induce fungal growth in the yeast tank. Simultaneously, the fermentation tanks are filled with liquefied mash, and gluco-amylase is added to the process to begin the process of saccharification, i.e., to hydrolyze dextrin to fermentable sugars. When the propagated yeast is ready, it is added to the fermenter and during this step, yeast ferments glucose to ethanol and carbon dioxide (Kwiatkowski et al. 2006). A typical corn-to-ethanol plant has three or more fermenters operating in batch mode in staggered cycles of the 48 to 72-hour fermentation process. This anaerobic process is operated at temperatures ranging usually from 30 to 35 ºC, since higher temperatures can induce the decline of yeast metabolic activity. Carbon dioxide from the process is removed through scrubbers (Kwiatkowski et al., 2006).

The fermented mash, known as “beer”, contains up to 16% of ethanol by volume, and presents non-fermentable solids from the corn and yeast cells. The beer is

pumped into continuous distillation columns, where ethanol is separated from the solids and most of the water as an azeotrope. The first distillation columns produces ethanol in concentrations normally ranging from 60-80% in volume, and water from this process is usually recycled to the alpha-amylase tank. The ethanol-water mixture is distilled up to the 95.6% azeotropic-concentration point on the second column, where it is sent to a molecular-sieve system, producing anhydrous ethanol (Belyea et al., 2004).

The other fraction of the beer, containing the bottom solids from the distillation, is called whole stillage. Whole stillage processing is later described with further details on this text.

### 1.3. Nutrient mass balance

Material balances determine quantities of raw materials required and products produced in a system, and are a basis for process design. For a complete process, balances of individual units determine stream flows and compositions. The conservation equation in material balance is presented as the following:

Material out = Material in + Generation – Consumption

This approach has been used in many systems to determine nutrient pools, i.e., steps in a process with higher concentration of a studied nutrient (Breen, 1990). High concentration of certain minerals in DDGS has direct impact on its value, and especially, on the animal feed characteristics it will present. These will not cause only nutritional disorders, like diarrhea, but also excessive mineral concentration in wastes, which are often applied as fertilizers. Phosphorus has been reported as of high environmental concern, since the high concentration of

this mineral in DDGS, is known to be overdosed and the manure generated contains P in concentrations above the land retention capability (Hao et al., 2009).

While nitrogen (N) and phosphorus (P) in manure are valuable nutrients for fertilizers, they are also the most concerning environmental pollutants from animal manure. Increased nitrogen and phosphorus excretion due to the incomplete digestion of DDGS is one of the current barriers in widespread land application of manure (Hao et al., 2009).

Swine manure, when used as nutrient source for crop production, has an imbalance of nitrogen and phosphorus, relative to the crop nutrient needs. On the other aspect, commercial inorganic phosphorus is completely produced from nonrenewable resources (mined from phosphate rocks) and predictions about their depletion time imply this to happen in the next century (Abelson 1997) Bioavailability of P in animal feed is another important factor with direct effect on the retention in ingested feeds by non-ruminant animals (e.g. poultry and swine), since their ability to digest phytate is low (Rausch and Belyea 2006). Availability of P in organisms directly correlated to its chemical forms, being classified as orthophosphate P, phytate P, and remaining P, which is a sum of all P present in nucleic acids, proteins, and phospholipids (Maga 1982). Orthophosphates can be digested by non-ruminants, as poultry and pigs, as well as by ruminants, as cattle (Cheng and Hardy 2002).

### 1.4. Importance of coproducts from ethanol production

Since starch present in corn is converted into glucose, which is converted during

the fermentation process to ethanol and carbon dioxide, the nonfermentable materials are consisted on corn kernel proteins, fibers, oils, and minerals. These are used to produce a few key products with particular interest of animal feeding industries.

DDG (i.e., Distillers Grains without added solubles) is a common byproduct, which can correspond up to 25% of distiller's grains sales (Rausch and Belyea 2006). However, due to the large amount of solubles produced (processed as thin stillage after the first centrifugation), and their composition that add value to the distillers grains, DDGS is often marketed as the most appropriate feeding material derived from coproducts. DDGS are dried to approximately 10% moisture content to ensure an extended shelf life, and it is sold to local livestock producers or shipped by truck or rail to various domestic and international destinations. Currently DDGS typically contains about 10% fat, 30% protein, up to 12% starch and 40% neutral detergent fiber (Rausch and Belyea 2006).

### 1.5. Recovery of products and value addition to ethanol coproducts

In a typical dry-grind plant, coproduct recovery starts with whole stillage, which accounts for the bottom fraction of ethanol distillation from fermented mash. Whole stillage, upon leaving distillation, contains 6-16% of total solids, and is a hot, acidic, and viscous fluid, with limited shelf life. Whole stillage is usually dried for easier handling, storage, and end use. The most common practice to handle whole stillage and transform it into a stable product consists on a series of separations, first using a solid-liquid separation. The solid fraction from this separation is known as wet distiller's grains (WDG), and the liquid fraction, which

contains about 90 to 95% of moisture, is thin stillage. Thin Stillage (TS), after some treatments is dried to produce Condensed Distillers Solubles (CDS), to a moisture content of 50 to 75%. This fraction is then combined with WDG to produce a nutrient-rich material, which is dried in order to produce DDGS (Reis et al. 2017). There are several reasons for this drying to occur in several steps instead of drying WS directly to DDGS. The first one is that a significant portion of TS is used as source of water and nutrients to the cooking step, which yields to water and thermal energy savings. This step is known by the term backsetting, and is often coupled with a series of anaerobic digestors. The second factor is related to energy savings during different drying processes: removing water in a dryer can use as much as 500% of the energy necessary to operate an evaporators, since evaporators allow reuse of thermal energy. The third reason is that CDS, containing 25 to 50% of solids, is a very viscous material, and this increased viscosity imposes additional difficulty in homogeneous drying; however, when CDS is mixed with WDG, the mixture is easier to dry (Reis et al. 2017).

### 1.6. Thin Stillage

Thin stillage contains about 3 to 4% suspended solids and 2 to 5% (wt/wt) dissolved organic materials with the typical value of 100 g $L^{-1}$ COD, which is the organic material available for value-added products (Mitra et al. 2012). Considering the high volume, high carbon content and low value of the thin stillage (TS) generated in the dry milling ethanol production process, microbial production of important commodity chemicals and high-value products can

enhance the economy of the corn ethanol industry. Thin stillage can be a heavy pollution source if it was not evaporated and dried to serve as animal feed. TS was found to contain glucan oligomers (12.4 g $L^{-1}$) and glycerol (14.4 g $L^{-1}$) as major components along with monomer sugars (glucose, xylose, and arabinose), and various minerals and amino acids as minor components (Kim et al. 2008). The evaporation process to condense TS is the second most energy-intensive step in the corn ethanol production, after the ethanol distillation (Rausch and Belyea 2006). Also, having TS as part of DDGS also brings many issues in the animal feed materials (Khan et al. 2013). Recent studies shows that the TS can be an effective medium as a carbon or nutrient supplement in microbial fermentation for the production of polyunsaturated fatty acids (Liang, 2012), 1,3-propanediol (Khan et al., 2013), fungal polysaccharides (Hsieh et al. 2005), algal biomass (Mitra et al. 2012), malic acid (West, 2011), butanol (Ahn et al. 2011) and also reusing thin stillage for ethanol production (Pejin et al., 2009). The growth and production of useful products from the low value thin stillage would certainly generate revenue for the corn growers and the distiller's.

Alkan-Ozkaynak et al. (2010) developed the first studies reported in utilizing chemical coagulation and flocculation as a treatment for thin stillage, in order to reduce P levels. According to Alkan-Ozkaynak et al. (2010) the majority of P and solids in thin stillage are in the dissolved form (<0.45 μm), which, if centrifuged, would yield a solid fraction with high crude protein and low P concentration. This solid fraction has similar properties as WDG and can be directly utilized as

animal feed additive. On regards to removal of colloidal inorganic and organic P, lime ($CaCO_3$) achieved up to 85% of P removal. This removal, on their study, was further evaluated using cationic and anionic flocculants, and it was found that anionic polymers provided bigger and more stable flocs. Their optimized results, of adding, 1.2 g of lime and 10 mg of PAM (anionic polymer) per liter of thin stillage, would cost about $53,000 per million gallons of ethanol produced, when their study was done.

A further study published by Alkan-Ozkaynak & Karthikeyan (2011) evaluated the performance of Anaerobic Digestion on lime-treated TS. They cited as major findings that treated-thin stillage appears to be a superior substrate to microorganisms, when compared to raw TS, resulting in rapid production of high levels of biogas. Their study also indicate that the digestate would be suitable, in quality terms, for recycling as process water.

### 1.7. Phosphorus in ethanol coproducts

Technologies for phosphorus removal in ethanol coproducts can be divided into biological, chemical, and physical methods. Biological methods are those that remove phosphorus by utilizing biomass to uptake this mineral as an essential nutrient for their growth or by any means of sorption into their biomass. Some examples of these methods include membrane bioreactors, biological filters, and activated sludge. Physical methods are those based on separation using any retaining solid, usually with mechanical aid, and can be cited as membrane filters and adsorbents. Chemical methods are related to change in the compound structure and stability, and include precipitation (including coagulation and

flocculation) and crystallization.

Chemical precipitation is perhaps the earliest developed and most common used method in water and wastewater treatment. In such method, phosphorus, as phosphate, is precipitated by forming precipitates with salts of multivalent metal ions. The three most used are calcium, ferric and aluminum ions. The solubility of calcium phosphates has been well described in the literature, and it forms precipitates as dihydrogen phosphate ($Ca(H_2PO_4)_2$), dicalcium phosphate dehydrate ($Ca(H_2PO_4)_2.2H_2O$), calcium hydrogen phosphate ($CaHPO_4$), calcium hydrogen phosphate dehydrate ($CaHPO_4.2H_2O$), and hydroxyapatite $Ca_5(PO_4)_3.OH$, depending on pH values.

Ferric and aluminum salts also precipitate orthophosphate through a coagulation process. Coagulation is a physical chemical method that involves the destabilization of dissolved and colloidal impurities, which, through flocculation, form large aggregates which can be removed in subsequent clarification or filtration processes. Ferric and aluminum salts react with water molecules and form a series of products, which have a large surface area and carry positive charges. These hydrolysis products have large surface area and carry positive charge. For example, when ferric coagulants added to water in the pH range of 6-8, five monomers ($Fe^{3+}$, $Fe(OH)^{2+}$, $Fe(OH)_2^+$ , $Fe(OH)_3$ (molecule) and $Fe(OH)_4^-$ ), a dimer and trimer ($Fe_2(OH)_2^{4+}$ and $Fe_3(OH)_4^{5+}$), and a solid precipitate ($Fe(OH)_3$ (s)) are. These ferric polymeric species can be represented as $[Fe_m(OH)_x]_n^{(3m-x)n+}$. They precipitate phosphate through charge-neutralization,

complexation, and/or adsorption (Wilfert et al. 2015).

### 1.8. Phytate

Phytic acid (known as inositol hexakisphosphate (IP6), inositol polyphosphate, or phytate when in salt form), discovered in 1903, is the main storage form of phosphorus in many plant tissues in many seeds, grains, and cereals (Maga, 1982). The structure of inositol can bind up to six phosphate anions. According to Ravindran et al. (1995), phytate is present in levels ranging from 50% to 80% of the total phosphorus present in corn.

The catabolites derived from phytic acid are called lower inositol phosphates. Some examples are Inositol penta- (IP5), tetra- (IP4), and triphosphate (IP3) (Maga, 1982). Phytate has a strong chelating potential, being able to bind to minerals present in grains such as calcium, magnesium, iron, zinc, which can contribute to mineral deficiency when these are used as feeding materials (Graf et al. 1987)

Phytate has a wide range of applications in many fields, especially due to its strong chelating property. Examples are the utilization as antioxidant agent in the food industry, since it is able to suppress oxidative reactions catalyzed by iron, which improves food shelf life, and can help reduce the risk of some inflammatory bowel diseases (Graf et al., 1987); the utilization of lower inositol phosphates by medicine-related industries, since it is known that these can have a function in second messenger transduction systems (Fox & Eberl, 2002), as well as the use in decreasing effects of gastritis, duodenum inflammation, and diarrhea (Hambidge, 1992); the use as anti-corrosion agent due to its strong

metal-complex potential energy, which provides a non-poisonous and easily-controlled galvanization process (Neevel, 1999); and the use by plastic manufacturers, since it is known to prevent blocking of copolymers (Xu et al. 2005).

The worldwide production of phytate has increased 28.8% over the last 5 years, achieving a 2015 production of about 5600 tons. Over the same period, China contributed to about 47% of the total production. Phytic acid chelates with cations in the digestive tracts of animals to form insoluble complexes. Once these complexes are formed, the phosphorus present in it cannot be utilized by animals, unless phytate is hydrolyzed by microbial phytases. Spiehs et al. (2002) reported a phosphorus overdose in DDGS, having reached levels as high as 10 g P $kg^{-1}$ in dry basis, which is much higher than the requirements of most ruminant animals (Rausch and Belyea, 2006). The largest portion of organic phosphorus in DDGS, as in corn, is found as phytate. This molecule cannot be directly assimilated by non-ruminant animals because the lack of microbial phytases in their digestive track (Cromwell et al., 1995). Feeding non-ruminants with phytate-rich materials, such as DDGS, will further increase the phosphorus in manures, which are often applied as fertilizers by farmers (Spiehs et al, 2002). This overdose of phosphorus in soil typically exceeds the soil binding capacity of phosphates and other forms of phosphorus ions, potentially resulting in high levels of phosphorus in runoff water. The major environmental impact of such effect is the eutrophication in surface and underground water bodies (Rausch

and Belyea, 2006; Noureddini et al., 2009). Phillippy et al. (1987) evaluated the potential of industrial yeasts in hydrolyzing phytate during ethanol fermentation, and reported that these are able to produce phytase. These enzymes are able to degrading phytate to the forms of inositol mono-, bis-, tris-, tetra-, and pentakisphosphates, as well as inorganic phosphate. However, according to Liu and Han (2011), due to fermentation conditions often applied, about 40% to 50% of phosphorus in the fermentation mash usually remains as phytate.

Phytate is reported to be unaivailable biologically to monogastric animals, but it can be hydrolyzed by ruminants due to the presence of phytase in their rumen. In ruminants, the bioavailability of phosphorus from cereals, such as corn, usually is greater than 50%. Using an artificial rumen technique, Raun et al. (1956) demonstrated that rumen microorganisms hydrolyzed calcium phytate, suggesting the presence of phytase. However, in monogastrics, it has been shown that natural phytate is poorly digested by growing swine. Similar results have been reported by numerous researchers when feeding phytate in cereal grains to various species of poultry, having first been reviewed by Nelson (1967). The chelating property of phytate in digestive tracts not only affect phosphorus bioavailability, but also can yield deficiency of certain minerals, such as zinc, magnesium, calcium, and iron. It also has been reported to interact with enzymes, such as trypsin, pepsin, *alpha*-amylase, and *beta*-galactosidase, resulting in a decrease of their activity.

Certain technologies for phytate removal from cereals and grains have been

developed mostly to increase the nutritional value and the bioavailability of nutrients linked to phytate. Mechanical processes, such as milling, have been reported as efficient phytate removal techniques in germ-rich grains, such as corn, in which 89% of phytate is found in the germ portion (Yi et al., 1996). In wheat, rye, and rice, phytate is concentrated in the outer layers, and hence normal milling has been reported to remove most of phytate from these cereals. In soybeans and dry beans, most phytate is associated with the protein bodies in the cotyledons and cannot be removed by milling. Brooks and Morr (1984) applied alkaline extraction to soybean and was able to remove circa 80% of the phytate present in it. There are reports on strategies using selective extraction and differential solubility methods to precipitate and remove phytate from soybean, and soy products (Dilger and Adeola, 2006)

The selective extraction procedures reported in the literature are highly pH-dependent. Most of them are at pH 5.5 or less, since it has been reported that similar procedure at higher pH values tend to form undesirable substances, such as lysinoalanine. At low pH values, a common procedure has been the combination of excess calcium ions, which compete with protein cationic sites for phytate binding. Deshpande & Cheryan, (1984) evaluated this approach on dry beans and reported a reduction of about 60% in phytate content.

Another significant approach that has been developed is the use of membranes to separate phytate from proteins and other components in legumes and cereal extracts. Membrane processes are highly dependent on the molecular size. As

an alternative to calcium precipitation, these post-extraction techniques have been reported to separate up to 72% without appreciable loss of proteins in soybean extracts (Cheryan & Rackis, 1980). Laboratory scale experiments indicated that ultrafiltration techniques can be a promising approach for selectively removing phytate (Lai et al., 2013). Ultrafiltration offers the advantages of mild and easy-controllable processing conditions and selectivity. The drawback is the high operating and capital cost of its implementation. Okubo et al. (1975) prepared a low phytate soy protein isolate by first dissociating phytate from protein by chemical treatment and then passing the dissociated phytate through a 30 kDa molecular cutoff ultrafiltration membrane. This approach, based on an EDTA chemical treatment, has been reported to recover up to 95% on a phosphorus basis.

### 1.9. Separation by Ion exchange processes

Phytate is found at low concentrations on ethanol coproducts, which imply that the current technology of producing phytate from cereals not to be convenient. If phytate were precipitated from thin stillage or other coproduct stream, excessive large amounts of such stream would need to be processed and extremely large filters would be necessary to recover the solids. In order to purify the precipitate solids and to produce phytate salt free from protein and other contaminants, large amounts of wash water would be needed.

A technology that has been applied to purify ionic compounds found in solution, such as antibiotics, aminoacids, and other compounds of high-value, is the use of ion exchange systems. Ion exchangers are water-insoluble solid substances

which can take up ions from an electrolyte solution and release other ions of like charge into the solution in an equivalent amount (Inczédy, 2013). The ion exchange process in the case of anion exchange can be described by the equation:

$$R\text{-}A + B^- \leftrightharpoons R\text{-}B + A^-$$

Where R is the anion insoluble in water and capable of ion-exchange, and $A^-$ and $B^-$ are the univalent anions taking part in the ion exchange. This process is reversible, i.e., they can be reversed by suitably changing the concentrations of ions in solution. Ion exchange is in many respects analogous to adsorption processes, having as major characteristic difference the stoichiometric relationship present in ion-exchange, while in pure adsorption systems, there is no ion release into the bulk solution.

The most common type of ion exchanger of high specificity used in the industry is the synthetic resin ion exchanger. These usually have a high capacity, i.e., number of exchangeable ions per unit weight of resin, good resistance in both chemical and mechanical respects, and retain their capacity after constant use. These materials consist of a large organic molecular network to which active groups able to dissociate are fixed. Anion exchangers contain basic groups as active groups. Under the category of anion exchangers, two main groups are defined by ion exchangers' manufacturers: weak base, having primary, secondary or tertiary amine groups as active exchange sites, and strong base, which contain quaternary amine groups as exchange groups (Inczédy, 2013).

Ion exchange resins have been used in chemical and biochemical applications for decades, being basis for an important product purification technique (ion exchange chromatography), and vast pharmaceutical, metallurgical, and agricultural applications (Zagorodni, 2006).

### 1.10. Research significance and objectives

The main objective of this thesis is to increase the profitability of ethanol through recovery of compounds and processing on the “waste” streams. The interest in providing higher economically sustainable options to ethanol plants has driven much of the research done in this field. The purpose of this research is to provide additional revenue to ethanol plants, and as side effect, to diminish the environmental impacts of ethanol production. Ethanol plants are owned by a significant number of farmers in the Midwest, employing more than 8,000 people in Minnesota. The opportunity of retooling this traditional, yet dynamic industry, is the key driver of this research.

# CHAPTER 2: LITERATURE REVIEW - NEW TECHNOLOGIES IN VALUE ADDITION TO THE THIN STILLAGE FROM CORN-TO-ETHANOL PROCESS

## Outline

Grain-to-ethanol production has increased steadily in the United States in the past years, which resulted in a remarkable increase in the availability of coproducts. Dry-grind ethanol production from corn grain is the most common processing method, and the downstream processing conditions applied concentrates the nutrients in the remaining one-third of the corn kernel into distiller's feeds. The ethanol coproducts have traditionally been a commodity livestock feed or feed supplements as they contain desirable nutrients. Distillers' grains and the nutrient rich wastewater after the distillation and separation of solid materials, called thin stillage, are typically mixed, dried, and sold as dried distillers' grains with solubles. A portion of thin stillage is normally recycled as backset water, while the remaining goes through a series of evaporations. Evaporating a large amount of water from thin stillage is an energy-intensive process and recycling the thin stillage may lead to the environmental concerns regarding nutrient overdosing in livestock feed operations. There are several other industrial processes to utilize thin stillage, such as distiller's corn oil extraction, anaerobic digestion, and secondary fermentation. Recently, promising results have been reported on the production of important commodity chemicals, extracting high-value products and energy production from thin stillage. This review article provides an overview on the new processes and products via valorization of thin stillage by innovative technologies that are being currently

developed. The new applications of thin stillage discussed herein could open new opportunities for the ethanol plants and ethanol researchers by increasing the revenue and simultaneously reducing its environmental footprint.

*Key words: Thin stillage, Value-added utilization, Ethanol, Bioprocessing*

## 2.1. Introduction

Ethanol has been produced for fuel in the United States since mid-1970's and it currently is the largest ethanol producer in the world, accounting for over half of global production in 2015 (Figure 1). Ethanol gets its economic value from the energy it contains as well by its additive value. It has been used as a gasoline additive because it contains more oxygen than gasoline, causing the blend to burn cleaner, and has a higher octane value than gasoline. Nowadays, many other factors drive the economic and environmental sustainability of ethanol, mostly due to the importance of coproducts, especially distiller's grains.

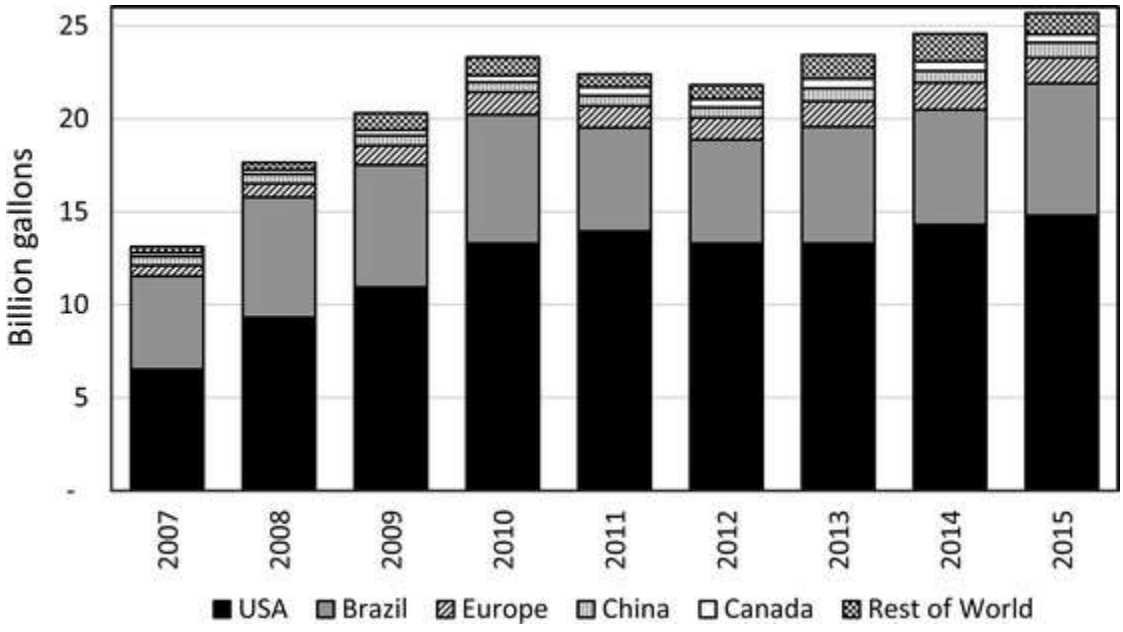



Figure 2.1: Global ethanol production by region. Adapted from RFA (2017)

Commercial production of fuel ethanol involves breaking down the starch present in corn to simple sugars, followed by the bioconversion of sugar molecules to ethanol *via* yeast. The downstream processing involves the recovery of ethanol by distillation and the residual coproducts are further processed to serve as animal feed. Dry-grinding accounts for over 80% of the total ethanol production in the United States, and it differs from the wet-milling process by the absence of an initial steeping operation to the corn grain where most of the coproducts of interest are separated before the fermentation process. In the dry-grind process, the residual components are separated after the fermentation and distillation. The remaining nutrients from the ethanol distillation are the raw material for producing coproducts. Since starch present in corn is mostly converted into glucose, which is then converted during the fermentation process to ethanol and carbon dioxide, the non-fermentable materials constitute corn kernel proteins, fibers, oils, and minerals (Kim and Dale 2008). The coproducts are mainly used as animal feeds, and ample research has been done in improving their quality to have animal feed production to be as important as the fuel ethanol production. The recovery of coproducts is one of the key drivers for the economic sustainability of ethanol manufacturing, which is especially marketed as livestock feed, can account for nearly 25% of the total revenue for some ethanol plants (Hill et al. 2006).

As of 2016, there were 231 operating ethanol plants in the United States, with a production capacity of approximately 15 billion gallons on a yearly basis (Pouliot

and Babcock 2017). Taking into consideration that the US corn ethanol industry increased eight-fold between 2000 and 2012, it is crucial that even minor modifications in ethanol processing can have significant environmental and ecological impacts. Fuel ethanol plants are developing new technologies to sustain the fluctuating market not only in the upstream and fermentation processes, but also in the coproducts downstream flows. Recent upgrades involving energy generation using anaerobic digestion, distiller's corn oil extraction, and production of 1.5-generation ethanol, have been developed with the technological focus on coproducts. One of the coproducts, Thin Stillage (TS) is the key parameter for all these successful modifications, which will be discussed in this review.

Among the two major processes used to produce ethanol in the United States, the wet-milling process starts with a steeping operation on the corn grain, which separates it into starch, germ, and fibers (Wang et al. 2008). In the dry-milling process, corn flour is produced through a grinding process, which is processed without separation of starch (Kim and Dale 2008). The dry-milling process, featuring with lower capital investment and an easier operation and logistics, therefore dominates the recent installations. The corn-to-ethanol process on a dry-grind plant is summarized through the following operations from the upstream through the first downstream step: milling, mashing, cooking, liquefaction, saccharification, fermentation, distillation, solids separation, evaporation, and $CO_2$ scrubbing (figure 2) (Belyea et al. 2004).

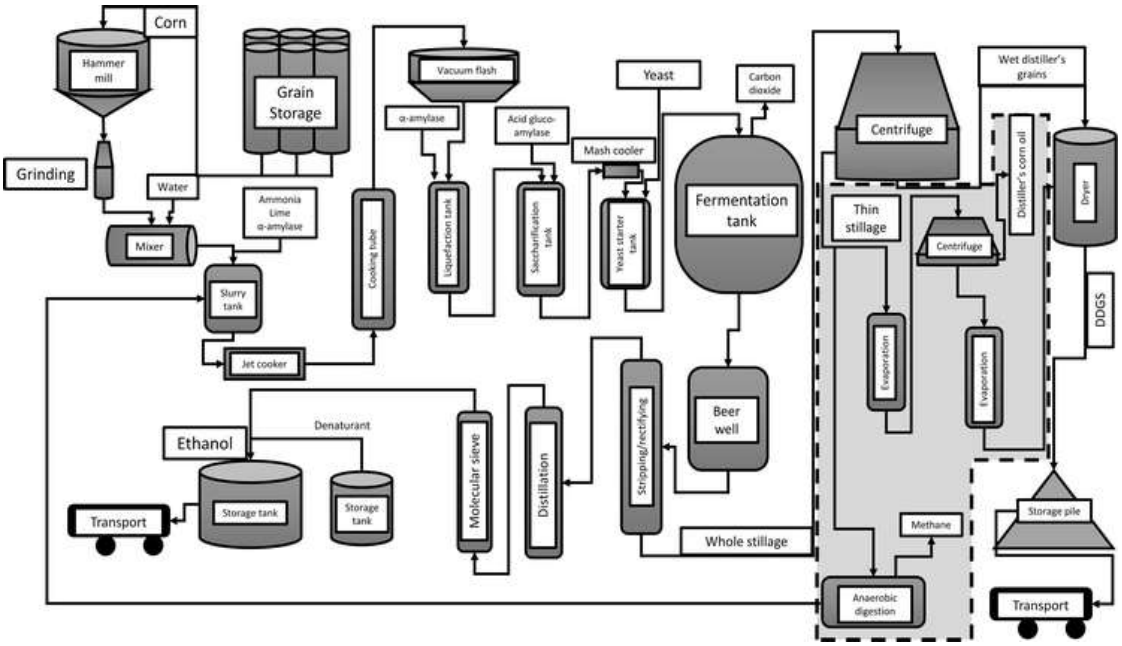



Figure 2.2: Summarized flowsheet of a typical dry-grind ethanol plant. Adapted from Rosentrater and Muthukumarappan (2006)

In the first step of dry-grind processing, the corn is hammer-milled and ground to a fine powder called the meal. Corn meal is mixed with water, derived from corn grains, makeup water, recycled water, water condensate from evaporators, and $CO_2$ scrubber water, producing corn mesh. Alpha-amylase is added to the slurry mixer to start the degradation of starch to dextrin (Belyea et al. 2004). Corn slurry is then cooked using a hydro-heater. Steam enters the cooking system to heat the slurry and a pressure drop of up to 2.8 atm is applied to facilitate the mechanical shearing of starch molecules. The slurry is heated to 90-120 ºC, and it is held at this temperature for up to 10 min, with a saturation pressure of about 2 atm. The high temperature and pressure in this cooking step help reducing the

bacterial levels in corn mash (Belyea et al. 2004).

The cooked mash is liquefied, which partially converts the gelatinized starch to soluble dextrin, decreasing the viscosity significantly. Additional alpha-amylase is added to this step since the cooking process denatures most of the previously-added enzymes (Belyea et al. 2004). Yeast propagation process starts aerobically to induce cell growth in the yeast tank. Simultaneously, the fermentation tanks are filled with liquefied mash, and gluco-amylase is added to the process to being the process of saccharification, i.e., to hydrolyze dextrin to fermentable sugars. When the propagated yeast is ready, it is added to the fermenter and during this step, yeast ferments glucose to ethanol and carbon dioxide (Kwiatkowski et al. 2006). A typical corn-to-ethanol plant has three or more fermenters operating in a batch mode in staggered cycles of the 48 to 72-h fermentation process. This anaerobic process is operated at temperatures ranging usually from 30 to 35ºC since higher temperatures decrease the yeast metabolic activity. $CO_2$ from the process is removed through scrubbers (Kwiatkowski et al. 2006). The fermented mash, known as “beer”, contains up to 16% of ethanol by volume, and presents non-fermentable solids from the corn and yeast cells. The beer is pumped into continuous distillation columns, where ethanol is separated from the solids and most of the water as an azeotrope. The first distillation columns produce ethanol in concentrations normally ranging from 60-80% in volume, and water from this process is usually recycled to the alpha-amylase tank. The ethanol-water mixture is distilled up to the 95.6% azeotropic-

concentration point on the second column, where it is sent to a molecular-sieve system, producing anhydrous ethanol (Belyea et al. 2004).

In a typical dry-grind plant, coproduct recovery starts with whole stillage, which accounts for the bottom fraction of ethanol distillation from the fermented mash. Whole stillage from distillation column contains 6-16% of total solids, and is a hot, acidic, and viscous fluid, with limited shelf life. Whole stillage is usually dried for easier handling, storage, and end use. The most common practice to handle whole stillage is to transform it into a stable product which consists of a series of unit operations, first using a solid-liquid separation. The solid fraction from this separation is known as wet distiller's grains (WDG), and the liquid fraction, which contains about 90 to 95% of moisture, is TS (Belyea et al. 2004). TS is dried to produce Condensed Distillers Solubles (CDS), to a moisture content of 50 to 75%. The drying is done in several steps instead of drying WS directly to Distiller's Dried Grains with Solubles (DDGS) as (i) a significant portion of TS is used as the source of water and nutrients to the cooking step, which yields to water and thermal energy savings. This step is known by the term backsetting and is often coupled with a series of anaerobic digesters. (ii) The second factor is related to energy savings during different drying processes: removing water in a dryer can use as much as 500% of the energy necessary to operate evaporators, since evaporators allow reuse of thermal energy. (iii) CDS, contains 25 to 50% of solids, is a very viscous material, and this increased viscosity imposes additional difficulty in homogeneous drying; however, when

CDS is mixed with WDG, the mixture is easier to dry. CDS fraction is then combined with WDG to produce a nutrient-rich material, which is dried in order to produce DDGS (Belyea et al. 2004).

Table 2.1.: Composition of thin stillage by cellulosic biomass compositional analysis and forage/feed nutritional analyses (Adapted from Kim et al. (2008))

| Cellulosic biomass compositional analysis | |
|---|---|
| Dry matter | 7.7 |
| Glucose (g $L^{-1}$) | 0.9 |
| Glucan (oligosaccharide, g $L^{-1}$) | 12.4 |
| Xylose (g $L^{-1}$) | 0.7 |
| Xylan (oligosaccharide, g $L^{-1}$) | 3.7 |
| Arabinose (g $L^{-1}$) | 0.4 |
| Arabinan (oligosaccharide, g $L^{-1}$) | 0.5 |
| Lactic acid (g $L^{-1}$) | 16.8 |
| Glycerol (g $L^{-1}$) | 14.4 |
| Acetic acid (g $L^{-1}$) | 0.3 |
| Butanediol (g $L^{-1}$) | 1.9 |
| Ethanol (g $L^{-1}$) | 0.6 |
| Forage/feed nutritional compositional analyses | |
| Compositional analysis | |

| | |
|---|---|
| Dry matter | 6.2 |
| Crude protein | 1.3 |
| Crude fat | 1.3 |
| Carbohydrates | 2.8 |
| Ash | 0.8 |
| **Forage analysis** | |
| Gross calories (kcal $kg^{-1}$) | 28 |
| ADF (Acid detergent fiber) | 0.1 |
| Cellulose | 0.1 |
| Starch | 0.5 |
| **Mineral analysis** | |
| Calcium (ppm) | 31 |
| Phosphorus | 0.1 |
| Potassium | 0.2 |
| Magnesium | 0.1 |
| Sulfur | 0.1 |
| Sodium | 0.1 |
| Chloride | 0 |
| Iron (ppm) | 8 |
| Manganese (ppm) | 2 |

All values are % dry basis except where otherwise noted.

TS processing is problematic and it has significant negative impact on the DDGS as animal feeds. TS is produced at scales up to 15 times the volume of ethanol produced and concentrating TS to CDS is one of the most energy intensive processes in the corn ethanol bio-refinery. TS processing comprises a series of evaporators to remove water and produce CDS. Due to the composition of TS especially with proteins, fat, fiber and other carbohydrates, evaporator fouling occurs during its concentration steps. The consequences of fouling include decrease capital and operating costs, and indirect environmental footprint of cleaning chemical disposal. A study conducted by Kim et al. (Kim et al. 2008) reports the averaged composition of three different ethanol coproducts – DDGS, WDG, and TS. Kim et al. (Kim et al. 2008) provided a collaborative work by three research groups, and details the composition as proteins, fat, and ash, as well as detailed analysis on cellulose, xylan, arabinan, and starch contents (Table 1). Non-starch polysaccharides have been analyzed since they are not readily bioavailable to monogastric animals, and are of limited value as a feed component for cattle. TS, as a liquid feed, has been recognized as excellent energy and protein sources for several different animals, such as growing and lactating cattle. TS can be used as a partial or complete replacement of water. When TS is fed with poor quality feeds, it acts as an energy and protein

supplement. In well balanced diets, TS improves feed efficiency by reducing dry matter intake. Additional consideration should be given to lysine supplementation since it tends to be deficient in corn TS. However, with massive inclusion of DDGS as animal diet, for instance, 30%-40% in hog diet, TS components such as phytate and phosphorus, potassium, sulfur etc, may pose over application potential and contribute to digestion issues. Therefore, TS has been widely explored as a separate source to produce valuable chemicals for different applications. The purpose of this review is, thus, to present current research being made in the process of integrating a TS-based biorefinery into the corn-to-ethanol industry (figure 2.3).

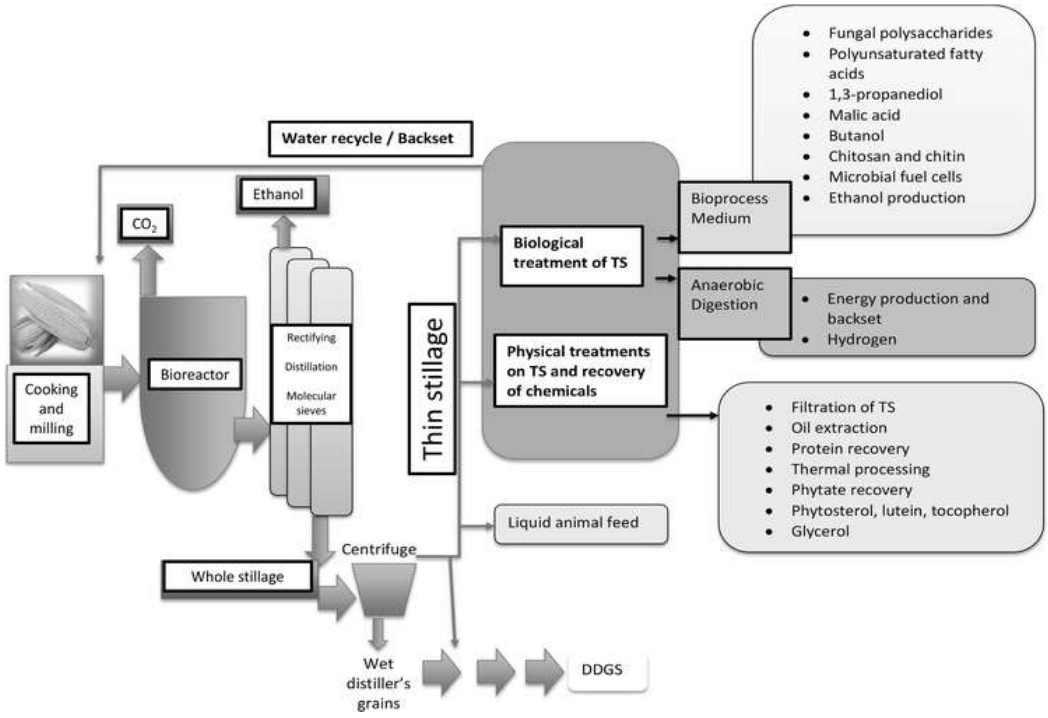



Figure 2.3.: Process flowsheet to describe various applications of thin stillage

## 2.2. Extraction of value chemicals from TS

TS is a high-moisture content mixture, accounting for most of the dissolved particles present in the corn-to-ethanol downstream process. The fermentation process produces significant amount of by-products, such as glycerol, and is responsible for lowering the pH of corn mash. The complex mixture present in TS, if separated, can be a key factor in developing sustainable bio-refineries. Nowadays, extraction of distiller's corn oil from TS has been cited as a factor that defines a feasibility factor to survival of the biodiesel industry in the United States, as well as significant increase in the economic revenue for an ethanol plant.

### 2.2.1. Oil extraction

The oil (crude free fat) content is increased from 4% in corn to about 14% in DDGS, like many other minerals during the corn processing. Although fat increases the energy density of DDGS for livestock, it may have a direct impact on the milk production by dairy cattle and an undesirable softer texture of pork bellies (bacon) in DDGS-fed swine(Saunders and Rosentrater 2009). Unlike front-end corn degerming, i.e. prior to fermentation, oil recovered from dry-grind TS is not suitable for feed-use. However, tail-end recovery, i.e. post fermentation, is expected to require much less capital and lower operating costs due to the fermentation conditions applied (Moreau et al. 1999). Despite recovery of oil in whole stillage being possible (Noureddini et al. 2009), the downstream corn oil recovery in dry-grind ethanol plants is usually accomplished by placing the

separation step within the TS evaporators. TS is sent to a first evaporator, oil is recovered, and then TS is further concentrated to distiller's solubles. The most desirable post-fermentation step to remove oil is during the centrifugation of TS. The current dry-grind centrifugation operation conditions make about 50% of the total oil to go to TS (Shevkani et al. 2014).

The fat content of DDGS from traditional corn ethanol facility can range from 8.8 to 12.4%, significantly higher than corn (Shurson et al. 2012). However, the majority of the ethanol industry is currently extracting oil to produce reduced-oil (4 to 8%) DDGS (He et al. 2014). The extracted oil can be used as raw materials for biodiesel production, but the majority of the oil is added in the chicken feed to increase the energy content. DDGS are consistently high in unsaturated fatty acids with nearly 80% of fatty acids are unsaturated containing 25.7% oleic acid and 55.35%, linoleic acid(He et al. 2014). According to Gunstone (Gunstone 2011), corn oil contains 59.6% linoleic acid, while soybean oil contains 51.0% linoleic acid. Even though there are reports showing that the oil extraction did not significantly affect the energy and feeding value in swine diet (Gunstone 2011), the economic value of corn coproducts after oil extraction is declining and there is tremendous need to develop ways to enhance the feeding value of reduced-oil DDGS to maintain market demand from the swine and poultry industries (Parsons et al. 2006). High-end applications based on the lipid-rich characteristics of TS include its use as anti-corrosion agent (Kharshan et al. 2016).

Oil extraction from TS has been developed in different stages of processing (figure 4). TS in ethanol processing has its solids concentrated during the evaporation steps, including the oil phase. The specific gravity of TS increases during the heat treatment, which has an impact on the separation of the aqueous and organic phases. The evaporation step usually starts at a heat pre-treatment at 100 ºC for undisclosed amount of time (Majoni et al. 2011b). One approach that has been reported is the separation of oil before the evaporation stage using a centrifuge (Prevost and Hammond 2003), which is not a commercial success. It has been reported that this approach does not produce usable oil, but an undesirable emulsion phase that requires further processing. Moreover, the volume of TS is generally up to 10 times greater than CDS, i.e., syrup, which requires considerable capital to acquire the number of centrifuges required. Another patented approach (Bento and Fleming 1993) describes the use of filters for removing substantially all solids and recovering lactic acid and glycerol from TS without the need for evaporation. The most common practices industrially process the condensed TS through a centrifuge, which sends back the de-fatted syrup to the evaporation process (Majoni et al. 2011b). This removal process includes oil but despite eliminating a step in the conventional process, this method would result in a more complicated arrangement due to the multiple filtration units required to separate the solids. The elimination of the evaporator in the majority of existing plants is unlikely and uneconomical, and its removal may cause significant changes in DDGS formation. The utilization of separation aids,

such as precipitated and hydrophobic silica, has been described as a further alternative to decrease the need for additional separation units, which can be added on the inlet or outlet of an evaporator (Lewis and Shepperd III 2016). The key issue regulating the successful application of chemical aids on distiller's corn oil recovery is the temperature of the process, since the surface properties of the aid compound may be negatively impacted by an increase in temperature. Hydrophobic silica, for example, decreases oil yield if the treatment temperature is above 90 ºC (Lewis and Shepperd III 2016). The use of non-ionic surfactants (Tween® 80 and Span® 80) has also been tested, which overall improved up to 10% more oil when compared to control experiments (Fang et al. 2015). Due to the complex composition of TS, the utilization of chemical aids for separating oil may be a potential future commercial application, since current centrifugation units are not able to destabilize the oil-in-water emulsified droplets, thus, not recovering all the possible distiller's corn oil present in TS.

The recovery of oil from TS after evaporation using a disk stack centrifuge has been disclosed by Winsness and Barlage (2008). This process includes heating the TS at a temperature greater than 100 ºC at a pressure greater than its vapor pressure, followed by a cooling phase which helps to separate the oil from TS. The traditional use of continuous centrifuges and three-phase decanter centrifuges, to remove oil does not provide yields as high as those by disk stack centrifuges, since the latter apply higher centrifugal forces and those are

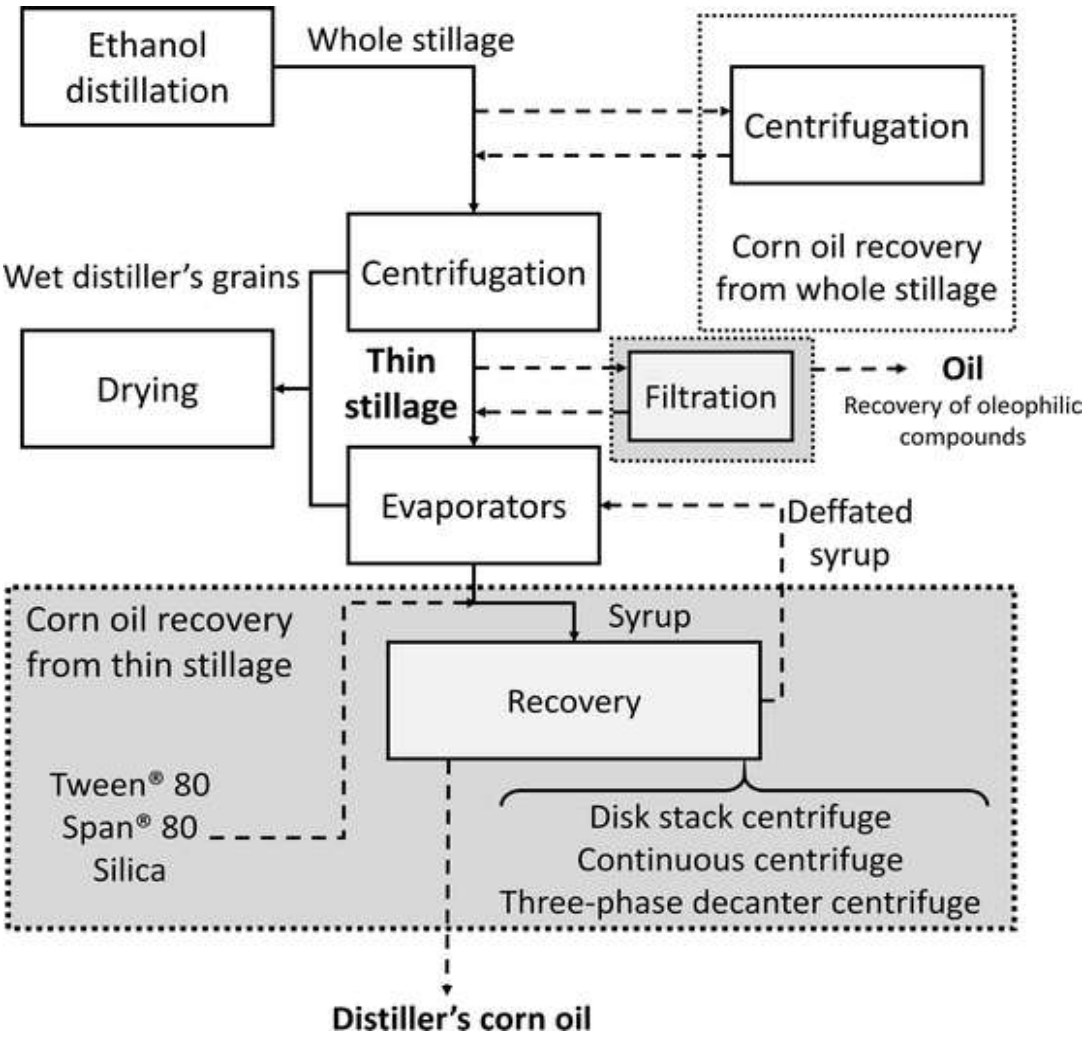


Figure 2.4.: Technical applications to remove distiller's corn oil from ethanol coproducts. Adapted from Moreau et al. (2013)

are able to remove bound and emulsified oil (Moreau et al. 2013). Wang et al.( 2008) developed a study to evaluate the corn breaking methods and its impact on TS oil concentration. The laboratory simulation showed that the oil partition in TS positively correlates to dry matter yield of the TS. Grinding alone has limited effect on oil partition, and flaking makes oil more extractable. Flaking-extrusion released the highest amount of free oil among all physical size reduction treatments studies, and oil released by flaking was less extractable after fine grinding, probably due to the mixing of the free oil with the hydrophobic components in the endosperm particles. They also conclude that in order to maximize the oil partitioning in liquid phase during dry-grind corn ethanol fermentation, two aspects must be considered: (1) enhancement of oil release from corn germ particles, and (2) avoidance of released oil to be absorbed by the endosperm or corn meal. Their results showed that extrusion of corn stillage showed promising results to as a way for corn pretreatment to maximize oil recovery; however, the meal fines in the liquid must be greatly reduced.

It is important to note that distiller's corn oil can be used to produce biodiesel and other renewable products (Arora et al. 2008). Current amounts are at about 1.2 billion kg of distiller's corn oil extracted from thin stillage (Shurson 2016). If 70% of the oil can be recovered from all dry-grind corn ethanol plants in the United

States, an additional of 2 billion L of corn oil could be annually produced, which is nearly equivalent to the biodiesel production in the US (Arora et al. 2008). The environmental impacts of a potential hybrid energy system, combining distiller's corn oil extraction from the dry-grind ethanol process, with the transesterification-based reaction and product recovery to generate biodiesel, are known to be significant. Within a process-level allocation approach, the ethanol life-cycle greenhouse gas emissions are at 46 g $CO_{2eq}$ $MJ^{-1}$, greatly lower than the estimated 62 g $CO_{2eq}$ $MJ^{-1}$ at a marginal distiller's corn oil level. Therefore, not only a greater use of distiller's corn oil might impact the economic displacement of traditional vegetable oil, but also, it may decrease the environmental greenhouse gas emissions of dry-grind ethanol.(Wang et al. 2015)

### 2.2.2. Protein recovery, degradation and utilization

The study of protein recovery from TS has not been explored elaborately. Arora et al. (Arora et al. 2010) evaluated the potential of recovering proteins using micro and ultrafiltration. Microfiltration was performed using a 0.1 μm pore size stainless steel membrane, which ultrafiltration experiments were performed in batch using three regenerated cellulose membranes, with molecular weight cut offs of 1, 10, and 100 kDa. The results indicate that for a 23.5% protein TS, microfiltration is able to increase the protein content to 27.6% on the retentate. Ultrafiltration at 1, 10, and 100 kDa increased the protein to levels of 32.2%, 29.0%, and 28.4%, respectively. Even though, there has been significant research done in recovering zein from ethanol coproducts, there is a limited

number of reports on utilizing the liquid fractions for such recovery (Anderson and Lamsal 2011). Using sequential filtration systems, Ratanapariyanuch et al. (Ratanapariyanuch et al. 2016) concentrated TS-based proteins into a slurry, with protein content of 50% on a weight basis. The potential commercial application of filtration devices to extract proteins has been tested, which, with a coupled drying device, is able to produce a stable product with low water activity ($a_w$=0.3), and protein levels as high as 60% (Ratanapariyanuch et al. 2016). Another trend for this research is to utilize proteins during the corn ethanol fermentation itself and the strategy has been successfully applied in many production facilities. Protease enzymes, either from bacterial or fungal sources, can be used at various stages of ethanol production. Typically, proteases, which hydrolyze proteins to peptides and free amino acids, can increase the utilization of corn protein by the yeast, decreasing the amount of external nitrogen addition, releasing more starch materials for hydrolysis, and therefore further improving the yeast fermentation of sugars. The process of adding proteases to ethanol fermentation increases the kinetics of the process, and decreases glycerol concentration (Harris et al. 2014). In addition, they can also facilitate the process of dewatering solids from whole stillage (Gautam 2011). Looking at the end product as DDGS, the addition of proteases during the milling step may induce higher starch recovery, and significantly lower starch and protein content in DDGS, but with a greater concentration of amino acids. The high fiber content of protease-modified DDGS would potentially result in a good animal feed for

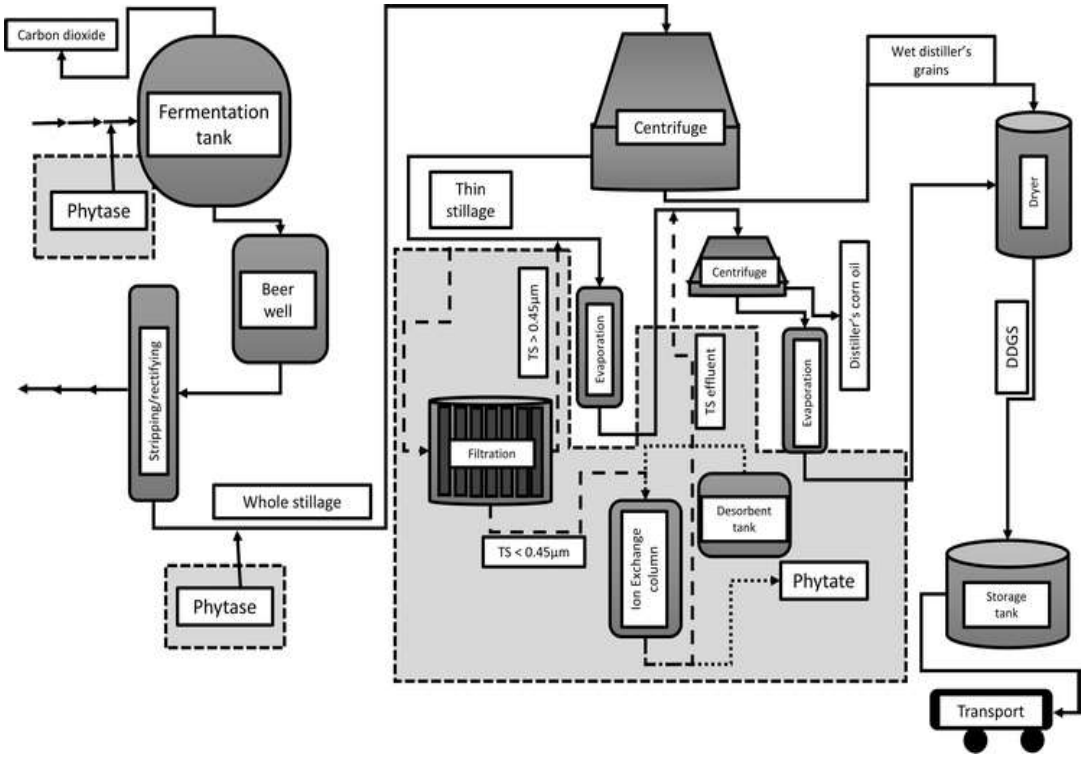
ruminants. A study reported by Vidal Jr et al. (Vidal Jr et al. 2009) has found that when using protease, the residual starch in endosperm fiber after fermentation was reduced by 8% and 22% in dry-grind with raw starch fermentation and dry fractionation with conventional fermentation, respectively. Also, the addition of protease in TS may alter the viscosity, making the drying process through evaporators less energy intensive (Vidal Jr et al. 2009). The application of proteases may not only potentially benefit fermentation needs, but also provide greater oil yields in TS, since this class of enzyme is able to degrade hydrolyze oleosins, known to encapsulate oil in corn kernels (Huang 1996; Majoni et al. 2011a). Under the right conditions, TS could be used as a potential source for manufacturing a high-protein material, or could have its rheology enhanced due to the use of proteases (Harris et al. 2014).

### 2.2.3. Phytate recovery and degradation

Cereals and legumes contain significant amounts of phosphorus in the form of phytic acid (*myo*-inositol hexakisphosphate). Phytic acid, also known popularly by its salt form, phytate, serves several physiological functions and also influences the functional and nutritional properties of such crops by complexing with proteins and essential minerals. The catabolites derived from phytic acid are called lower inositol phosphates; some examples are Inositol penta- (IP5), tetra- (IP4), and triphosphate (IP3) (Maga 1982). Phytate has a strong chelating potential, being able to bind to minerals present in grains such as calcium, magnesium, iron, zinc, which can contribute to mineral deficiency when these are used as feeding materials (Graf et al. 1987). Corn is a crop with the major

concentration of phytate, and the dry-grind processing concentrates phytate to about threefold in DDGS (Almeida and Stein 2010; Maga 1982). Phillippy et al. (Phillippy et al. 1987) evaluated the potential of industrial yeasts in hydrolyzing phytate during ethanol fermentation, and reported that yeasts were able to produce phytase. These enzymes are able to degrade phytate to the forms of inositol mono-, bis-, tris-, tetra-, and pentakisphosphates, as well as inorganic phosphate. However, according to Liu & Han (Liu and Han 2011), due to the fermentation conditions often used, about 40% to 50% of phosphorus in the fermentation mash usually remains as phytate. Phytate accounts for 60-82% of the total phosphorous in corn (Ravindran et al. 1994). During dry-grind processing of corn into ethanol, the ratio of phytate P to total P decreased to about 46% upon yeast fermentation and remained similar in the following processing streams, indicating that phytate might be degraded by yeast (Liu and Han 2011). The remaining phytate P in coproducts is not digested by non-ruminants and excreted in manure. The phosphorus bioavailability of DDGS is only about 60% for poultry (Kim et al. 2008).

Figure 2.5.: Extraction of phytic acid and phytase addition to ethanol coproducts




streams

Phytate, if extracted from TS, could be highly employed by sectors of the food, textile and chemical industries. Due to its strong chelating potential, phytate provides inoxidizability effects and is often used as food additive and preservative, as well as being a strong anti-corrosion agent. Interests from the pharmaceutical industry are related to the prevention of renal calculi, and some types of cancer, among others (Maga 1982). A novel technology to extract phytate from TS has been disclosed using ion exchange (Fig. 5). Ion exchange methods are efficient in recovering phosphorus compounds and purify phytate from TS (Hu and He 2016; He et al. 2017). This extraction has no evident adsorption on nutritional ingredients of stillage, such as fat, fiber, proteins and

residual starch, and can bring additional revenue to the bioethanol industry by extracting and purifying this value-added product. The ion-exchange process is highly selective for phytate and does not interfere with other nutritional aspects of TS, such as amino and fatty acid composition. Using BioRad AG 1-X8 anion exchange resin for phytate extraction, and NaCl as desorbent, a maximum yield of 1.85 g of calcium phytate per kg of TS processed was achieved (He et al. 2017).

imilarly, addition of phytases (myo-inositol hexakisphosphate phosphohydrolases or phytate phosphatases) in corn mash aims at breaking down phytate to release free phosphate (Fig. 5). It has been reported that incubating corn starch with alpha-amylase in the presence of phytic acid has resulted in reduced activity of amylase (Deshpande and Cheryan 1984), and the degradation of starch was reduced by 50% at the highest tested condition of phytic acid (Yu et al. 2014). Phytases have been conventionally supplemented during feed formulation with DDGS for diets of poultry (Martinez-Amezcua et al. 2006), swine (Hill et al. 2008), and fish (Cheng and Hardy 2002) to release the phytate phosphorus, thus making it bioavailable to the animal. The increase in bioavailability reduces the amounts of supplemental P required in the feed as well as reduces the amounts of undigested and excreted P in manure (Selle and Ravindran 2007). Methods have been developed to add phytases on the slurry before liquefaction in the upstream processing (Shetty et al. 2008) and on whole stillage (Noureddini and Dang 2009). Some reports states that the addition of phytase in ethanol

production process offers economic and environmental benefits, as it not only creates low-phytate coproducts, but also solves the waste-disposal problem and improves the overall efficiency of ethanol production (Khullar et al. 2011; Shetty et al. 2008). A study developed by Noureddini and Dang (2010) evaluated the use of commercial phytases (Natuphos® and Ronozyme®) to degrade phytate in TS. They observed that at a concentration of ≤4.8 FTU mL−1 TS for Natuphos phytase (≤48 U mL−1 TS for Ronozyme phytase), and within mesophilic temperature ranges (≤60 °C), complete phytate hydrolysis could be achieved within 1 h of enzymatic treatment. It was observed that the reduction in phytate P was accompanied by the increase in inorganic P, maintaining total P relatively unchanged (Noureddini and Dang 2009; Noureddini and Dang 2010). Luangthongkam et al. (2015) observed that addition of such hydrolytic enzymes can also enhance partitioning of solids and oil into TS, and the production of DDGS with the lower amount of non-digestible carbohydrates. Such modifications can play a significant role in the design of new ethanol plants, as well as on the DDGS market to monogastric animals, which have poor digestion capabilities of phytate and fibers.

### 2.2.4. Extraction of glycerol and other valuable chemicals

Different techniques have been explored to recover glycerol from TS in the ethanol industry, which include vacuum distillation (Yen et al. 2012), azeotropic distillation (Overheul et al. 2014), reverse osmosis (Russo Jr and Kim 1996), and nanofiltration (Bleyer and Czartoski 2016). However, most of these techniques provided a separation of glycerol, polyols, organic acids, and traces of sugars

from the TS stream, thus requiring further additional energy-intensive treatment to yield pure products.

A study developed by St. Julian et al. (1990) evaluated the effect of recycling TS in the overall concentration of glycerol. According to their results, each fermentation lead to a 0.4% increase in glycerol, achieving as high as 2.1% in the beer on the fifth recycle. Cheryan and Parekh (1995) developed a method for separating glycerol and organic acids from TS using a sequence of electrodialysis, which removed most of the organic acids from the glycerol-rich TS stream, and selective crystallization, which precipitated lactic acid from TS. This selective precipitation was made using a metal oxide, which complexed the lactic acid. This complex was separated using an acidic compound, generating lactic acid in its un-dissociated form.

A physical separation process has been developed for the separation of organic acids from glycerol, other polyols, sugars, and other uncharged compounds that result as by-products from ethanol fermentation (Kampen 1993). According to Kampen (1993), using an electrochemical-based apparatus, the stillage from an ethanol plant could be clarified (e.g. by microfiltration) to remove suspended particles that might otherwise plug up the narrow feed channels in the electrodialysis (ED) stack. In addition, any soluble salts in the stillage will also be transported into the product stream, depending on the ion mobility. Thus the ED product stream will contain few inorganic salts, resulting in high levels of glycerol, but it will also contain other non-charged components such as sugars that remain

unconverted during the fermentation. The commercial feasibility of this process will further depend on finding a suitable market for these byproduct components. The purity of the glycerol from ED, and that of the organic acids from the precipitation step may not be sufficient to compete with other commercial sources of these compounds, and they may have to be processed further. Glycerol production and price has been driven by the biodiesel industry, responsible for 67% of world production in 2014 (Luo et al. 2016). Glycerol recovery from TS is found at significantly lower concentrations and yields than those of the biodiesel industry, however, it still can be beneficial for food and beverage, pharmaceuticals and textiles industries, or as a substrate for production of high-value chemicals.

With the advances in recovering oil from TS as discussed previously, there is a potential market for phytosterol production from the co-product (Winkler et al. 2007). Phytosterols are triterpenes, with a similar structure to cholesterol, found ubiquitously in plants. They exist as free and acylated sterols, as acylated sterol glycosides, or as esters to ferulic or p-coumaric acid. In vegetable oils, they are found mostly as free or acylated sterols. Besides the importance of regulating several biochemical pathways in plant cells, they represent a high-value commodity for the pharmaceutical industry, as an added product to low density lipoprotein (LDL)-reduction formulations. The level of total phytosterols in post fermentation corn oil has been reported to be at around 5% on a weight basis (Moreau et al. 2010). Despite most of the work is done on characterizing

phytosterols in DDGS, it can be assumed that the levels of phytosterols have significantly decreased in this product with the oil extraction (Moreau et al. 2010). The presence of tocopherols and tocotrienols are higher in distiller's corn oil when compared to traditional corn germ oil, and are of similar level as those from solvent-extracted corn kernel oil (Moreau and Hicks 2005, 2006). Winkler-Moser and Breyer (2011) have noted that the significant amount of tocotrienols and tocopherols in distiller's corn oil may increase the oxidative stability of this material. Most of the work reported in the literature is found to be from the mid-2000s, when the first prominent works in oil extraction were taking place (Winkler et al. 2007).

Distiller's corn oil have a distinguishable amber color, which is greatly due to the levels of lutein and zeaxanthin (Moreau et al. 2007). These two classes of xanthophylls are important in human health, due to their requirement for a healthy macula region of the retina (Moreau et al. 2007). Lutein and zeaxanthin present in distiller's corn oil would not only make attractable for pharmaceutical extractions (Moreau et al. 2002), but also to poultry feed. Commercial production of these xantophylls are dependent on corn gluten meal or on marigold flower, and are often added to poultry rations to increase the yellow color of yolks and the yellow color of chicken meat (Moreau et al. 2007). Extraction of lutein and beta-carotene has been disclosed in a patent using adsorbent materials (Englert and Milos 2014). Therefore, distiller's corn oil derived from TS would also be suitable for potential studies in extracting and purifying these nutraceuticals.

## 2.3. Physiochemical treatment of TS

### 2.3.1. Filtration of TS

TS fouling has been evaluated in detail (Arora et al. 2010; Challa et al. 2015; Wilkins et al. 2006) and several solutions have been proposed. The effect of oil (Singh et al. 2001), phytic acid (Tian et al. 2015), pH (Wilkins et al. 2006), mixed carbohydrates (Challa et al. 2015) and other components on fouling have been evaluated. One of the proposed and most discussed solutions to this costly issue is the addition of a filtration step on TS processing (Arora et al. 2010). Several applications of membrane systems were identified and useful in dewatering processing streams. Despite several advantages, membrane filtration has not acquired widespread use in the corn refinery and dry-grind corn ethanol plants. One of the major problems associated with membranes is flux decline by pressure drop increase and membrane fouling. Accumulation of solute particles over the membrane surface causes the formation of gel layer or increasing osmotic pressure at the membrane-solution interface, resulting in a permeate flux loss. Irreversible fouling is due to blocking of pores and solute adsorption, and causes the loss in membrane permeability which cannot be regained completely. Several models have been proposed to predict permeate flux and are classified into three main categories: osmotic pressure controlled, thin film, and resistance in series (Arora et al. 2010). Osmotically controlled models and thin film models are suitable for filtration of aqueous solutions of low and high molecular weight solutes, respectively, and they present limited application (Cornelissen et al. 2008).

The model regarding resistance in series is used in various research areas to understand flux decline behavior and membrane fouling. The latter model includes reversible and irreversible components of fouling, and it is an effective approach that predicts short and long-term flux decline (Arora et al. 2010). Lapišová et al. (2006) studied ceramic three-channel membranes in various separation arrangements to treat TS. Their results by coupling an ultrafiltration membrane with kDa pore size with a 0.2 µm pore size membrane provided reduction of 20% in dry matter and COD. The outcome of their study shows that the filtered TS can be recycled at a rate of 75% up to 40 cycles without negatively affecting the ethanol production. According to their results, the composition of material had a great impact on membrane fouling. There is a significant lack of research in fouling behaviors, which limits the wide usage of membrane application in corn refineries (Lapišová et al. 2006).

Alkan-Ozkaynak et al. (2010) developed the first studies reported in utilizing chemical coagulation and flocculation as a treatment for TS, in order to reduce P levels. According to their study, the majority of P and solids in TS are in the dissolved form (<0.45 µm), which, if centrifuged, would yield a solid fraction with high crude protein and low P concentration. This solid fraction has similar properties as WDG and can be directly utilized as the animal feed additive. On regards to the removal of colloidal inorganic and organic P, lime ($CaCO_3$) addition in TS achieved up to 85% of P removal. This removal was further evaluated using cationic and anionic flocculants, and it was found that anionic

polymers provided bigger and more stable flocs. The optimized results, of adding, 1.2 g of lime and 10 mg of polyacrylamide (anionic polymer) per liter of TS, would cost about $53,000 per million gallon of ethanol produced when their study was done (Alkan-Ozkaynak et al. 2010). Alkan-Ozkaynak and Karthikeyan (2011) further evaluated the performance of anaerobic digestion on lime-treated TS. They observed that treated-TS appears to be a superior substrate for AD, when compared to raw TS, resulting in the rapid production of high levels of biogas.

The separation of thin stillage into a solids fraction and the water is of potential industrial interest due to a number of factors, including enhancement in the anaerobic digestion and superior quality for water recycled into the system (Alkan-Ozkaynak and Karthikeyan 2011), and the production of a co-product with higher bioavailability of amino and fatty acids (LoCascio and Dunbar 2014). The utilization of generally-regarded-as-safe (GRAS) chemical aids, e.g. GRAS anionic polymers, with a coupled dryer system generates a clarified TS and a dry feeding material in form of flocculated solids (LoCascio and Dunbar 2014).

### 2.3.2. Thermal processing

Combustion, gasification, and pyrolysis are three techniques that can be used to generate bioenergy from biomass feedstock. Biomass can be transformed into energy, primarily in the form of heat or electric power. Products produced from thermochemical conversion contain high concentrations of organic compounds, and thus are useful as concentrated sources of substrates for further utilization, i.e., conversion into fuels and chemicals (McKendry 2002).

Combustion occurs at a sufficient level of oxygen, and can result in flame temperatures around 2000 °C. This process can be accomplished in a variety of equipment: grate-fired, suspension, fluidized bed combustors, furnaces and boilers, etc. (McKendry 2002). Gasification converts biomass into a flammable gas using an oxygen-deficient atmosphere, generally at temperatures between 750 and 850 °C (Heidenreich and Foscolo 2015). It can be accomplished by a variety of gasifiers, which most commonly have updraft, downdraft, or fluidized bed configurations. The synthesis gas, commonly known as syngas, produced is often rich in hydrogen and carbon monoxide, and can be combusted and used to drive electric generation equipment or power boilers. The residual byproduct of gasification is ash, which is often used as fertilizer. Pyrolysis is a process in which the feedstock is heated in the absence of oxygen, generally between temperatures of 200–500 °C (Branca and Di Blasi 2015). It results in bio-oil, char and gases. Little research has been done on thermal conversion of TS into valuable products.

Delgado et al. (2015) developed a platform to utilize hydrothermal carbonization, which is a system that operates at moderate temperatures (175–250 °C) and pressure conditions. This process produces a carbonized charcoal-like material with improved C:O ratio, along with a liquid fraction, that can be easily separated by filtration, according to their study. HTC also uses around 80% less energy to turn TS into a shelf stable product (Delgado et al. 2015). Wood et al. (2013) processed TS using HTC and indicated that dehydration was the principal

carbonization process occurring during the reaction conditions. The C:O ratio obtained at the derived hydrochar indicate that this material could be used as carbon–neutral fuels still having coal-like heating values (Wood et al. 2013). Despite producing chars with low surface area (2.2 m2 g−1 for center point TS), post-thermal treatments can increase surface area, potentially increasing the value of TS-derived hydrochar (Wood et al. 2013). Recent utilizations of transformed hydrochars include supercapacitors for electrode materials (Falco et al. 2013), and sorbents for green-house gases, such as carbon dioxide (Sevilla et al. 2012).

A process developed by USDA researchers has established chemical, physical, and physicochemical methods for fractionating condensed fractions of TS (Milczarek and Liu 2015), which leads to new coproducts and a new strategy for dewatering CDS. On regards to the fractionated CDS, all the new fractions showed faster drying rates than the CDS as control when convectively dried at 60 °C, except for the glycerol-rich fraction. To further demonstrate the improved drying performance of the new coproducts fractions, they used a drum dryer to dry a protein-rich fraction and the control. The results show that while both materials could be dried to a range of endpoint moisture contents, the dried protein-rich fraction exhibited a broader range of water activity and lighter color than CDS (Milczarek and Liu 2015).

Reports on thermal processing of TS demand that the carbonization could improve the market share of coproducts, producing additional char, which acts as

a chemical absorbent and is a solid fuel with low ash and sulfur. Besides coproducts that could be directly applied as liquid fertilizer, other thermal processing techniques can yield better fractionation of TS. Dry TS could be readily turned into a shelf-stable, flaked product that could be marketed as a differentiated animal feed.

### 2.4. Thin Stillage as a feedstock for microbial cultivation

TS contains about 3 to 4% suspended solids and 2 to 5% dissolved organic materials with the typical value of 100 g COD $L^{-1}$, which is the organic material available for value-added products(Mitra et al. 2012). Considering the high volume, high carbon content and low value of the TS generated in the dry-milling ethanol production process, microbial production of important commodity chemicals and high-value products can enhance the economy of the corn ethanol industry. TS can be a heavy pollution source if it was not evaporated and dried to serve as animal feed. TS was found to contain glucan oligomers (12.4 g $L^{-1}$) and glycerol (14.4 g $L^{-1}$) as major components along with monomer sugars (glucose, xylose, and arabinose), and various minerals and amino acids as minor components (Kim et al. 2008). The evaporation process to condense TS is the second most energy-intensive step in the corn ethanol production, after the ethanol distillation (Rausch and Belyea 2006). Also, having TS as part of DDGS also brings many issues in the animal feed materials (Khan et al., 2013). Recent studies show that the TS can be an effective medium as a carbon or nutrient supplement in microbial fermentation for example, on the production of polyunsaturated fatty acids (Liang et al. 2012), 1,3-propanediol (Khan et al. 2013)

fungal polysaccharides (Hsieh et al. 2005), algal biomass (Mitra et al. 2012), malic acid (West 2011), butanol (Ahn et al. 2011) and also reusing TS for ethanol production(Pejin et al. 2009). The growth and production of useful products from the low value TS would certainly generate revenue for the corn growers and the distiller's. A summary of metabolites production and conditions used for TS as a production medium is given in Table 2. Fungal processes are known to be highly suitable to be developed using TS as medium, and can improve the economics of dry-grind ethanol by providing upgraded food-grade supplements. Dewatering fungal biomass grown on TS is highly dependent on the species and on the cultivation conditions applied. It is known, however, that filamentous species, such as *Rhizopus microsporus* var*. oligosporus*, can be easily separated using gravity screening with a filter centrifuge can remove up to 96% of free liquid, producing a solid-rich (30% on a weight basis) within a short period of time on an inexpensive process (Koza et al. 2017).

Table 2.2.: Examples of Thin stillage as bio-production medium

| **Metabolite** | **Strain** | **Thin stillage source** | **Mode of operation and Culture conditions** | **Yield / productivity** | **Ref.** |
|---|---|---|---|---|---|
| Butanol fermentation | *C. pasteurianum* DSM 525 | Prepared in the authors' laboratory or obtained from a local ethanol fermentation plant | Batch shake flask experiments [125-mL serum bottle containing 50 mL of medium; inoculated (1% v/v) and incubated at 37 °C with shaking (130 rpm)] | 6.2–7.2 g $L^{-1}$ [0.32–0.44 g butanol produced $g^{-1}$ glycerol consumed] | Ahn et al., (2011) |
| Food grade Malic acid production | *Aspergillus* strains | Not given | TS medium by filtering it through a Whatman No. 1 filter; Fungal inoculum (17% v/v) to 10 ml of sterilized thin stillage (pH 6.0) containing 9% (w/v) $K_2CO_3$ in a sterile 125 ml Erlenmeyer flask and grown for 192 h at 25°C (200 rpm). | Highest malic acid was produced by *Aspergillus niger* ATCC 9142 at 17 g $L^{-1}$. The highest malic acid yield (0.8 g $g^{-1}$) with *A. niger* ATCC 9142 | West (2011) |

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|---|---|---|---|---|---|
| | | | | and ATCC 10577 on TS medium | |
| Algae biomass | *Chlorella vulgaris* | Lincolnway Energy (Nevada, IA) | Bioscreen turbidimeter and 250 mL Erlenmeyer flasks (orbital shaker with 150-rpm agitation speed at 28 °C for 4 days, initial pH of 6.8; mixotrophic: light intensity of 880 lx. Heterotrophic cultivations: dark), then scaled up to a 6-L stirred bioreactor (4 days at 28 °C, 180 rpm and aeration rate of 0.7 SLPM (standard L per min; 2 vvm), constant pH of 6.8) | 9.8 g $L^{-1}$ biomass with the oil content of 43, % (w/w) | Mitra et al. (2012) |
| Eicosapentaenoic acid (EPA) production | *Pythium irregulare* | Lincolnway Energy (Nevada, IA) | Media containing different concentrations of thin stillage. Cells grown in 250-mL Erlenmeyer flasks containing 50 mL medium, and | With 50% thin stillage in a stepwise temperature shift culture process, the cell density reached 23 g | Liang et al. |

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|---|---|---|---|---|---|
| | | | incubated in an orbital shaker set at 200 rpm. The temperature (15-30 °C) was set to the desired levels based on experimental design. | $L^{-1}$ at day 9 with EPA yield and productivity of 243 and 27 mg $L^{-1}$ day. | (2012) |
| Microbial Polysaccharide production | *G. lucidum* | | Cultivated at 30 °C and 150 rpm for 7 days at different pH. Highest yield at pH 5, 60% thin stillage. | Highest cell concentration of 7.8 g $L^{-1}$ and polysaccharide production of 7.50g $L^{-1}$. | Hsieh et al. (2005) |
| Lipid and protein-rich fungal biomass | *Mucor circinelloides* | Lincolnway Energy (Nevada, IA, USA) | Shake flask experiments in 2 L Erlenmeyer flasks containing 500 mL of heat sterilized (121 °C, 15 min) TS. 10% (v/v) mycelial inoculum - incubated on a shaker at 150 rpm agitation speed and 37 °C for 2 days or longer. Optimized culture | Highest biomass yield obtained was 20 g $L^{-1}$ (dry), with a lipid content of 46% (g of oil per 100 g dry biomass). Supplementing TS with crude glycerol (10%, v/v) | Mitra et al. (2012) |

| | | | | | |
|---|---|---|---|---|---|
| | | | conditions scaled up to 6-L airlift draft-tube bioreactor with a 5 L working volume. Aeration rate of 7 SLPM (standard liters per minute; 1.4 vvm) was used. | led to 32% increase in cellular oil content. | |
| Algae-fungi co-culture | *Mucor circinelloides* UMN B34 – *Chlorella vulgaris* | Anonymous source | Shake flask experiments in 250 mL Erlenmeyer flasks containing 100 mL of heat sterilized (121 °C, 15 min) mimicked TS from CDS. Incubated on a shaker at 150 rpm and 28 °C for 7 days or longer. | Highest biomass yield of 6.61 g $L^{-1}$ of axenic fungal cultures and 9.072 g $L^{-1}$ of co-cultured biomass in 10x diluted CDS. | Rajendran et al (2016) |
| High-value fungal biomass | *R. oligosporus* (ATCC 22959) | Lincolnway Energy (Nevada, Iowa, USA) | Bench-top bioreactors (5-L): pH controlled at 4, 250 rpm agitation, 37° C, and the filter-sterilized aeration. Fungal spore inoculation of $1\times10^{7}$ spores. | Stirred bioreactors (5 L): 36 ± 4 g dry wt. $L^{-1}$ at 6 days and 1 vvm air flow rate | van Leeuwen (2010) |

| | | | | | |
|---|---|---|---|---|---|
| | | | 50-L reactor operated at similar culture conditions and inoculated with fungal mycelia (2% [v/v]). | 50-L cultivation : 28 g dry biomass $L^{-1}$ | |
| 1,3-propanediol | Wild-type and a recombinant strain of *Lactobacillus panis* PM1 | North West Bio-Energy Ltd. (Unity, SK, Canada) | Solid matter was removed from TS, and the resultant liquid part (liquid stillage; LS) was sterilized at 121 °C for 20 min. The fermentation tests were conducted at 30 °C for 6 days. *Batch, fed-batch, and pH-controlled batch fermentation*: Batch, or fed-batch fermentations were performed in 50-ml conical tubes containing 40 ml of fermentation media with overall molar ratio of glucose to glycerol of 0.37 in the both batch and fed-batch fermentation using LS. The glucose to | pH-controlled batch fermentation reduced the total fermentation period, resulting in the maximal 1,3-PDO concentration of 16.23 g $L^{-1}$ and yield of 0.72 g $g^{-1}$ in TS | Khan et al. (2013) |

glycerol molar ratio in batch fermentation using mMRS was 0.18. Batch fermentations (pH-controlled at 4.5 and agitation at 150 rpm) were performed in a 5-L fermentor with 0.61 mol glucose/mol glycerol ratio, and a 50-ml mid-log phase culture was used as inoculum.

### 2.4.1. Microbial polysaccharides

Fungal polysaccharides comprise a large group of biopolymers and have wide range of therapeutic use. Hsieh et al. (2005) studied the cell growth and polysaccharide production of *Ganoderma lucidum* on TS. According to their study, 60% TS was a suitable feedstock for growing the mycelia of *G. lucidum*, providing a cell concentration of 7.8 g $L^{-1}$. The reported polysaccharide production within the reported conditions, at pH 5, was 7.5 g $L^{-1}$. Their study also evaluated the effect of additive carbon sources, and molasses produced an increase in the cell concentration of 62.8%, and an overall increase in polysaccharides production of 49.2%. It was observed that both intracellular and extracellular polysaccharides showed a similar molecular weight distribution, ranging in the molecular weight from 10 kDa to 200 kDa. Polysaccharides of *G. lucidum* have been reported to have strong antitumor and anti-inflammatory properties (Mizuno et al. 1995). 1,3-β-D-glucan derived from *G. lucidum* cultivation is a promising type of carcinostatic agent, which has been reported to be useful in immunotherapy (Chan et al. 2005). Selenium-enriched polysaccharides from *Cordyceps sinensis* was produced using TS as substrate, and the fermentation parameters were optimized using response surface methodology (Yang and Zhang 2016). The bioactivity test of the selenium-enriched polysaccharides from *C. sinensis* in the rat based study shows that the polysaccharides can reduce blood glucose level and improve antioxidant capacity of rats with diabetes induced by streptozotocin (Yang and Zhang 2016) .

TS from rice wine distillery, was used as a feedstock to replace the high cost Hestrin and Schramm medium for bacterial cellulose production by *Gluconacetobacter xylinus* (Wu and Liu 2013). The bacterial cellulose production was significantly enhanced as 50 (v/v) % of Hestrin and Schramm medium was replaced with TS. The bacterial cellulose concentration of 6.26 g $L^{-1}$ after 7 d of static cultivation, which is approximately 50% higher in yield and 67% lower in feedstock cost than that could be produced using the conventional medium (Wu and Liu 2013).

### 2.4.2. Polyunsaturated fatty acids (PUFA)

PUFA are essential fatty acid required for maintaining good health. Liang et al. (2012) evaluated the production of eicosapentaenoic acid (EPA) by the cultivation of TS with Pythium irregulare. It was observed that 50% TS as culture medium can yield cell densities up to 23 g $L^{-1}$ in a 9-day cultivation. The EPA yield was 243 mg $L^{-1}$, at a productivity of 27 mg $L^{-1}$ $day^{-1}$. *Pythium irregulare* biomass comprised of 39% lipids, and its cultivation was able to deplete the nutrients in TS, removing all organic compounds. *Pythium irregulare*, can be considered a safe source of biomass and EPA-containing oil to be used in ingredients in supplements, food, feed, and pharmaceuticals (Liang et al. 2012). An increased concern in food and feed-producing fungi is the presence of mycotoxins. Wu et al. (2013) tested different *Pythium* strains, widely recognized as plant pathogens by infecting roots and colonizing the vascular tissues of various plants, like soybean and corn. The only species of the Pythium family known to infect mammals are *P. insidiosum*. *Mucor circinelloides* is a well-known

PUFA producing species (Reis et al. 2014a), and the growth of M. circinelloides in TS reported an increase in EPA concentration up to 2.6 times compared to a control system (Ravi 2014). According to the study, supplementation of glycerol at 20 g $L^{-1}$ is able to increase fungal oil up to 18%. Supplementation of nitrogen, at a concentration of 5 g $L^{-1}$ of ammonium sulfate, 5 g $L^{-1}$ of potassium nitrate and 0.5 g $L^{-1}$ of urea increased EPA content by 32, 28 and 22%, respectively. Mucor circinelloides and Trichoderma reesei grown on TS decreased the COD by levels >86% in 96 h, and achieved lipid accumulation in biomass at 22.11 and 9.82%, respectively (Bhanja et al. 2014). Yen et al. (2012) evaluated the use of a mixture of crude glycerol and TS on the cultivation of Rhodotorula glutinis. TS was used as an N replacement, and its use indicated a 27% increase in total biomass when compared to glycerol and standard medium. The use of the combination of these ethanol coproducts increases in linolenic acid ratio, comprising around 20% of the total lipids. The presence of glycerol in TS may significantly impact the production of PUFA by *Rhodotorula* strains, such as *R. mucilaginosa*, known for the high accumulation of docosapentaenoic acid, *R. aurantiaca*, *R. glutinis* and *R. gracilis* (Gientka et al. 2017). These strains are known to provide greater volumetric conversions of carbon source as glycerol towards PUFA accumulation than control tests with glucose (Gientka et al. 2017). Therefore, the natural presence of glycerol in TS derived from the ethanol fermentation may characterize TS as a suitable feedstock for PUFA-

accumulating species, potentially increasing the economic profit margins of ethanol producers.

### 2.4.3. 1,3-propanediol

1,3-propanediol is a building block in the production of many important polymers and are usually chemically synthesized. A bioprocessing platform was developed to produce the value added chemical 1,3-propanediol (1,3-PDO) from glycerol using a bacterial strain isolated from TS (Khan et al. 2013). The bacterial strain was identified as *Lactobacillus panis* by 16S rRNA, and its cellular characteristics showed that it was an aerotolerant acidophilic anaerobe which is able to grow over a wide range of temperatures. *L. panis* screened from TS was tolerant to high concentrations of ethanol, acetic acid, lactic acid, antibiotics, and sodium chloride.

Since glycerol is present in TS at about 10% of the total carbon output (Carere et al. 2008), it may be a suitable feedstock for 1,3-PDO production. Intensive research has been done within the last few years to increase 1,3-PDO levels, using both engineering and genetic strategies. Levels as high as 100 g $L^{-1}$ have been reported by Hirschmann et al. (2005). It has been observed on an exploratory study by Khan et al. (2013) that stored TS samples spontaneously produced 1,3-PDO, with a concurrent depletion of glycerol.

Demand for 1,3-PDO has been increasing significantly over the last decades due to its wide range of potential applications. This compound has direct applications in polyester and polyurethane synthesis, lubricants, solvents, and acts as an important precursor in the chemical and pharmaceutical industries. Thus, the

unrecovered and low-value glycerol naturally present in TS can yield an important economic stream for TS utilization.

### 2.4.4. Malic acid

Malic acid has many industrial applications, mainly in the beverage and food industry as an acidulant and flavor enhancer, chemical industries as a feedstock for chemical synthesisof polymalic acid, in metal cleaning, textile finishing, pharmaceuticals and agriculture (Chi et al. 2016). *Aspergillus* species have been reported as potential producers of malic acid. One of the first studies, developed by Peleg et al. (1988), had a malic acid concentration of 36.4 g $L^{-1}$ as the outcome, using a 100 g $L^{-1}$ glucose-based medium. The methabolic pathway of producing malic acid has been evaluated (Bercovitz et al. 1990), and the enzyme which regulates its production, pyruvate carboxylase has been engineered to induce *Saccharomyces cerevisiae* and *Escherichia coli* to higher production rates (Zelle et al. 2008; Zhang et al. 2011). An industrial waste stream (crude glycerol) as substrate was used to efficiently synthesis malate using *Ustilago trichophora* RK089 (Zambanini et al. 2016). The growth and production rate were increased by 2.5- and 6.6-fold, respectively by an adaptive laboratory evolution which is the highest titer reported for microbial malate production. The medium optimization increased the final titer, yield, and overall production rate to 196 g $L^{-1}$, 0.82 $g_{mal}$ g $_{gly}^{-1}$, and 0.39 g $L^{-1}$ $h^{-1}$, respectively.

Using TS as the substrate, West (2011) evaluated four different *Aspergillus* strains from *A. niger* and *A. flavus* species. The ATCC strains used were able to consume at least 94.5% of glycerol in the medium, and up to 62.7% of glucose.

ATCC 9142 strain was able to produce malic acid at a concentration of 16 $gL^{-1}$. West (2011) concludes his study describing the application potential of producing food-grade malic acid from TS. Even though malic acid has been extracted from apple juice (Peleg et al. 1988) most of its current production is through chemical synthesis to sustain a market of 40,000 annual tonne production (Zou et al. 2013).

### 2.4.5. Butanol

Even though ethanol represents the biofuel with the largest production volume and market share, and being largely used as a blend with gasoline, a number of drawbacks have been identified with ethanol use (Balat and Balat 2009). It has been proven that ethanol has a significantly lower energy content when compared to gasoline, that it is not amenable to pipeline distribution, and the amount used in gasoline is limited by performance factors (Jang et al. 2012). Many drawbacks have driven the exploration of advanced biofuels by companies who produce renewable fuels. Advanced biofuels are typically fuels with high energy content, and may include higher molecular weight alcohols (e.g. n-butanol and isobutanol) (Green 2011; Smith et al. 2010), and infrastructure-compatible hydrocarbons produced from thermochemical (e.g. Fischer-Tropsch based fuels) (Khodakov et al. 2007) or biochemical (e.g. fermentation-derived hydrocarbons) conversion pathways (Lee et al. 2015).

The rapidly increasing interest in biofuels development in the last decade has made the production of ‘bio-n-butanol’ being as the possible feasible alternative to ethanol. It is not completely compatible and fungible the way hydrocarbons

are; however n-butanol is considered a second-generation biofuel that is better for the existing infrastructure than ethanol. It has a higher heating value, is more hydrophobic, and it can be shipped via existing pipelines and distributed through existing petroleum infrastructure (Yilmaz et al. 2014). Some researchers claim that it will be one of the first biofuels to reach global markets(Pereira et al. 2015). Current EPA regulation allows the highest oxygen content in gasoline to be 2.7%, which allows up to 11.7% in volume of n-butanol to be blended with gasoline (Tao et al. 2014).

Fermentation of sugar-containing substrates to acetone-butanol-ethanol route is a process known for a century. *Clostridium acetobutylicum*, the bacterium used in the acetone-butanol-ethanol (ABE) route, has been used extensively for producing solvent in early-to-mid 1900s using molasses or corn mash (Kujawska et al. 2015). Current ABE fermentation processes use gene-modified *Clostridium* strains to utilize a broad substrate range, being able to metabolize glucose, fructose, mannose, sucrose, lactose, starch, and dextrin. Hexose sugars are metabolized via the Embden-Meyerhof pathway, and pentose sugars are metabolized via the pentose phosphate pathway (Kolek et al. 2016). *C. beijerinckii* is known to produce isopropanol in place of acetone, and has been recently used as the main form of biochemically producing isopropanol. *Clostridium* strains are spore-formers and obligate anaerobes. A study conducted by Ramey et al. (2007) has reported that higher n-butanol yields by separating

the fermentation into two separate steps – acidogenesis and solventogenesis, using different organisms during each phase.

Production of butanol using TS has been evaluated using *Clostridium pasteurianum* by Ahn et al. (2011) The composition of TS, especially due to pH and concentration of lactic acid, was found to be a positive factor in their study. Lactate/Lactic acid acted as a buffering agent, maintaining the pH within a range of 5.7-6.1 throughout *C. pasteurianum* fermentation, which provided an overall yield of 0.32-0.44 g of butanol produced per g of glycerol consumed. Previous results, reported by Biebl (2001) , show significantly lower yields, within the range of 0.31 g butanol $g^{-1}$ glycerol. In both studies, lactic acid was consumed subsequently to glycerol. Ahn et al. (2011) reported butanol production of up to 8.7 g $L^{-1}$ using the combination of glycerol and lactic acid. Though there are limited reports on platforms to produce hydrogen from TS, both these studies demonstrate the feasibility of cost-effective production using TS as a nutrient-containing medium with a pH buffering capacity.

### 2.4.6. Chitosan, chitin, and glucosamine

Chitosan is a natural and biodegradable polymer. It is mostly used as permeability control agent, as an adhesive, and in the paper industry as a sizing agent (Kumar 2000). Its surface properties have made this molecule as an interesting target for enzyme immobilization and as flocculation and chelating agent (Krajewska 2004). Industrial chitosan production involves processing of chitin that is treated as a waste from the seafood processing industry. Seafood wastes as raw material for chitosan production tend to produce heterogeneous

and inconsistent chitosan samples, mainly due to the different supplies of seafood wastes being seasonable and variable (Yokoi et al. 1998).

Several yeast and filamentous fungi have been reported to contain chitin in their cell and septa, including *Schizosaccharomyces pombe*, *Candida albicans*, *Saccharomyces cerevisiae*, *Mucor rouxii*, *Phycomyces blakesleeanus*, *Coprinus cinereus*, *Neurospora crassa*, *Trichoderma reesei*, *Rhizopus* spp., *Absidia* spp., *Mucor* spp., *Mortierella isabelina* and *Lentinus edodes (Wu et al. 2005)*. Fungal (*Aspergillus awamori)* cultivation in TS for chitosan production followed by an anaerobic process was studied by Ray & Ghangrekar (Ray and Ghangrekar 2016). Kinetic parameters were evaluated, and in 96 h of cultivation, up to 59.6% of COD and 70% of TOC were removed, and a chitosan recovery of up to 0.078 kg $kg^{-1}$ of dry mycelium was obtained. Chitosan was characterized using FTIR and XRD analyses, and the profile showed the high purity of the material recovered with dilute sulfuric acid. A combination of fungal fermentation with microbial fuel cells using TS as substrate has been disclosed to produce chitosan at levels of 0.7 g $L^{-1}$, with power generated from fuel cells at levels of 2.6 kW $m^{-3}$ (Ray and Ghangrekar 2015).

A patented invention describes the utilization of TS as feedstock for fungal cultivation, resulting in a material that can be used to produce high-quality glucosamine with low levels of impurities (Van Leeuwen et al. 2016). The glucosamine derived from chitin or chitosan from fungal cultivation is known to

have low ash content and being free of heavy metal contaminations, which are often toxic and present in traditional feedstocks. TS is a possible enhanced feedstock for chitin, chitosan, or glucosamine production via fungal fermentation when compared to crustacean-based extractions, since the produced material is known to be free of shellfish allergens (Van Leeuwen et al. 2016). The chitin and chitosan derived from TS does not constitute any relationship with animal products, therefore being suitable from vegetarian and does not conflict restrictions by religions that forbid consumption of shellfish (Van Leeuwen et al. 2016).

### 24.7. Ethanol

The potential of TS as culture medium to produce ethanol was evaluated (Pejin et al. 2009), based on the fact that there may be unconverted substances in the raw material used in the primary ethanol produce, and the TS would also contain all products of yeast fermentation and growth supporting chemical compounds. The high $BOD_5$, achieving up to 340 g $L^{-1}$, and high nitrogen levels help to cultivate *S. cerevisiae* in TS. Pejin et al. (Pejin et al. 2009) also evaluated the potential of TS recirculation in ethanol production, and indicate that up to 6 recycle times, which is the maximum numbers tested, may still provide an ethanol production of 96.36% compared to the theoretical conversion using 30% of recycled TS as the medium.

The study of TS as feedstock for ethanol fermentation has also been established by Ferreira et al. (2014) using *Zygomycetes* and *Ascomycetes* filamentous fungi. The production of ethanol from TS may be an interesting process from an

economical point of view since ethanol could be recovered without additional steps (Ferreira et al. 2014). The produced ethanol can be sent to the process, following the general stream towards the distillation column. This work also cites the production of high-quality fungal biomass, using *Zygomycetes (Rhizopus sp*.) and *Ascomycetes* (*A. oryzae, F. venenatum, M. purpureus* and *N. intermedia*). Ferreira et al. (2014) determined that an additional 5.5% of ethanol produced in a plant could be added by TS fermentation. With the aid of cellulase, second generation ethanol was produced using an innovative two-step cultivation using *N. intermedia* and *A. oryzea*, at concentrations as high as 7.6 g $L^{-1}$ (Bátori et al. 2015). S. cerevisiae is not a suitable strain for producing ethanol on TS, thus, other strains, which could generate as a by-product high-quality feed-grade biomass, would be preferable on the possible integration of first- and second-generation ethanol within a dry-grind plant. The utilization of *Clostridium thermocellum* on TS has also provided comparable ethanol production rates as those seen in a control medium (Islam et al. 2015).

Such fermentation technology would potentially assist other environmental and economic outcomes for an ethanol plant, such as the decrease of fermentation inhibitors in stillage water, which could be easily recycled into the fermentation tanks, as well as production of high-value biomass. Careful attention must be taken in account on the evaluation of batch-to-batch composition in order not to evaluate toxic chemical and inhibitory substances, since a few strains do not have high resilience for cell stress factors (Islam et al. 2015).

### 2.4.8. Microbial fuel cells (MFC)

Due to concerns regarding the pollution potential of distillery wastewater, estimated to be equivalent for a city of 500,000 people for a medium-sized ethanol plant (Wilkie et al. 2000), and presence of residual sulfate from cleaning practices (Liu and Han 2011), novel alternatives for treating TS have been developed. Recent studies disclosed the use of MFC to oxidize organic pollutants and sulfide in TS, providing an alternative to anaerobic digestion practices for energy generation (Ha et al. 2012; Lin et al. 2015) (figure 2.6). The high temperature of TS, usually processed within the range of 70 to 80 ºC, provides conditions favoring thermophilic MFC. The bacteria involved on these processes are called electrochemically active bacteria or anode-respiring bacteria, and have been previously disclosed by using acetate as the sole electron donor (Jong et al. 2006). Though there is still unclear information on the specific taxonomy of the bacteria involved within the process, TS has proven to be a successful substrate for MFC (Wrighton et al. 2008). Utilizing thermophilic MFC, the maximum current produced by a single cell by Ha et al. (2012) was 2.4 A $m^{-2}$ (838 ± 10 A $m^{-3}$) and power of almost 1.0 W $m^{-2}$ (342 ± 7 W $m^{-3}$), which are comparable or greater than reported values from wastewater-treating MFC (Ichihashi et al. 2011; Lu et al. 2009).

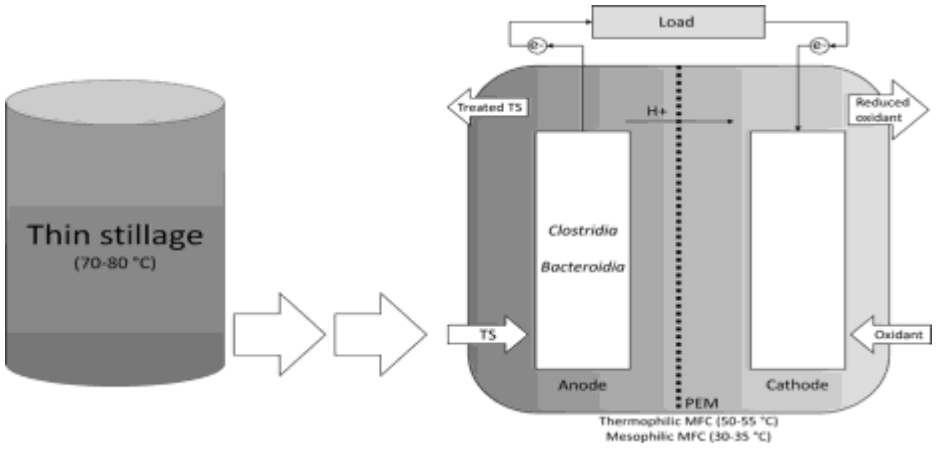



Figure 2.6.: Microbial fuel cell using TS as substrate. Adapted from Ha et al. (2012)

The use of mesophilic conditions to treat TS has also been disclosed, providing significant less current and power (0.35 A m-2, and 124 mW $m^{-2}$, respectively (Mohanakrishna et al. 2010)) when compared to thermophilic settings (Ha et al. 2012). The use of different electrodes on MFC using TS as substrate has effects on power generation. The use of graphite plate as anode has yielded 124 mW $m^{-2}$ of maximum power density, while interlaced carbon yarn provided maximum power of 348 mW $m^{-2}$ (Sonawane et al. 2014). A two-stage treatment of TS, consisting on fungal fermentation by *Aspergillus awamori*, nd MFC has also been disclosed, providing considerable reduction in COD by the fungal traetment of 70%, and an overall decrease in 99% of the total COD by the end of the process (Ray and Ghangrekar 2015). The two-stage treatment has proved to enhance the power generation in MFC by 2.9 times, removing all the suspended solids in TS

(Ray and Ghangrekar 2015). Therefore, the composition of TS coupled with its production volume in an ethanol processing facility could greatly impact the energy requirement for a plant, providing a substantial amount of energy via MFC utilization.

### 2.5. Anaerobic Digestion for energy production and backset

Anaerobic digestion (AD) consists of a series of complex processes involving several subgroups of anaerobic microorganisms, which leads to degradation of organic matter into reduced short molecules. In a typical ethanol plant, up to 20 L of TS can be generated per liter of ethanol (Rosentrater and Muthukumarappan 2006), and studies indicate that its balanced composition of COD, BOD, volatile solids and carbohydrates promote it as being a strong candidate for AD substrate (Eskicioglu and Ghorbani 2011; Eskicioglu et al. 2011; Nasr et al. 2012). Usually, due to the solids build up and toxicity to yeast caused by lactic acid, acetic acid, glycerol, and sodium, only 50% or less of TS is recycled as fermentation broth (Andalib et al. 2012). Applying digested TS, however, tends to improvements on water recycling within the ethanol production process. There is substantial energy production from the anaerobic digestion, in the form of methane gas. The production of methane gas has been reported as a way of reducing the usage of natural gas in the operations of distillation and drying, used on the ethanol purification and on DDGS production, respectively. According to Nasr et al. (Nasr et al. 2012), the natural gas consumption in an ethanol plant could be reduced to 43 - 59% with methane produced by AD. For a facility producing 360 million L ethanol a year, savings could be up to $17 million, according to their calculations.

It has been suggested that the effluent from digested TS could be employed by backset water without significant disruption in the ethanol production (Alkan-Ozkaynak and Karthikeyan 2011). The complex and rich mixture of TS contains nutrients and minerals, requiring only supplementation of few minerals, like trace cobalt, for long-term digestion (Agler et al. 2008).

Several researchers tried different technologies for anaerobic digestion on TS, varying basically on the temperature and on the number of stages of AD in the processes (figure 7). The differentiation on temperature usually comprises two classes of microorganisms, thermophilic and mesophilic (Kim et al. 2002). On regards to the number of stages, most studies reported in the literature indicate single or two-stage processes (Parawira et al. 2008). The first studies in analyzing corn stillages as the substrate for AD were done by Stover et al. (Stover et al. 1984). The mesophilic single-stage digestion trials, using a feedstock with 32 g of total solids $L^{-1}$ and 64.5 g total chemical oxygen demand (TCOD) $L^{-1}$, occurred in both suspended growth and fixed-film systems. Methane generation ranged from 0.22 to 0.33 $m^3$ $kg^{-1}$ COD removed, which, by that time, could replace 60% of the daily energy requirement for a bioethanol plant. A subsequent study by Lanting and Gross (1985) tested an upflow anaerobic sludge blanket (UASB) system, achieving 76% TCOD with a production of 0.33 $m^3$ $kg^{-1}$ TCOD removed.

According to Demirel and Yenigün (2002), a separation between acidogenic and methanogenic steps in anaerobic digestion provides enhanced stability to the

different groups of microorganisms and better process control. Thompson (Thompson 2008) highlighted that two-step systems can enhance net energy extraction from the AD conditions. Vinas et al. (1993) were among the first researchers to report a two-stage process for AD on TS. A methane production yield of 0.31 L $gCOD^{-1}$ removal was obtained, with an observable increase of 13% when compared to the single-stage process. Values as high as 89.8% of reduction of volatile solids were obtained in a 20-day CSTR, using an organic loading of 6.0 g COD $L^{-1}d^{-1}$ and also yielded 0.7 L CH4 $g^{-1}$VS. The production of VFAs was low, producing less than 200 mg $L^{-1}$ as acetic acid, which may be due to the low pH and close to zero alkalinity. Similar results were observed when applying a two-stage process, which evaluated an energy enhancement of 13%, when compared to the single-stage system(Nasr et al. 2012). The effect of ultra-sonication pretreatment on TS was studied to improve the operation, but it turns out to be a costly pretreatment with no significant benefits (Schaefer and Sung 2008).

The volatile solids reduction by anaerobic digestion lends to improved water recycling. Substantial energy potential is produced from anaerobic digestion in the form of methane gas. As ethanol production requires significant heat for distillation and drying, anaerobic methane production will reduce the use of natural gas within the ethanol production process and insulate the ethanol industry from volatile natural gas markets. Estimated natural gas displacement is 43 to 59% for a dry grind ethanol plant. Energy production value is estimated at

$7 to $17 million for a facility producing 360 million L ethanol $y^{-1}$(Nasr et al. 2012).

There are relatively few commercial two-stage anaerobic digestion units, despite their higher loading rates, improved stability and flexibility. Rapport et al. (Rapport et al. 2008) hypothesized that the added complexity and the expense of building and operating commercial two-stage systems have so far counteracted the yield and rate enhancements of such design. Several reactor designs, alongside the characterization of either single or two-stage, have been tested. A novel approach using high-rate anaerobic fluidized bed reactor (AFBR) have been used to treat TS with a TCOD level of 130 g $L^{-1}$ and 47 g $L^{-1}$ TSS. Promising results of removal up to 88% TCOD and 78% TSS have been observed (Andalib et al. 2012). The novelty of the work was characterized by the high rates of using 29 kg COD $m^{-3}$ and 10.5 TSS $m^{-3}$ and a hydraulic retention time of 3.5 days, which is significantly lower than 15, 20, and 30 days (Lee et al. 2011). Methane production rates of up to 160 L $d^{-1}$ at the steady state were observed. This production is equivalent to 40 L $CH_4$ $L^{-1}$TS and a 15.8 L gas L $reactor^{-1}$ $day^{-1}$ when compared to the reactor volume. A multi stage apparatus was designed to convert fermentation stillage into biogas (Friedmann 2015). The apparatus comprises of a separation unit for the separation of the fermentation stillage into a thin fraction and a thick fraction, at least one biogas reactor for fermenting the thin fraction and/or the thick fraction, and a storage tank. A return pipe from the second separation unit in the form of a nitrogen sink into the

bioethanol plant, to return the effluent process water for dilution of the reactor content in the biogas reactors (Friedmann 2015).

Among other designs for AD on TS, the utilization of anaerobic membrane bioreactors (AnMBR) for simultaneously treatment of TS and energy generation has been evaluated (Dereli et al. 2014). AnMBR providing a sustainable solution to sludge washout problems due to the biomass retention in the membranes (Dereli et al. 2014). Different retention times, from 20 to 50 days, have been evaluated, and higher organic loading rates up to 8.0 kg COD $m^{-3}$ $day^{-1}$ could be applied (Dereli et al. 2014). The utilization of AnMBR on high-lipid effluents, such as TS prior to distiller's corn oil extraction, can aid in solving the major drawbacks found in this particular type of AD, such as sludge flotation, biomass washout, granular sludge bed systems, and insufficient granulation. AnMBR utilization on high-lipid TS is still negatively impacted by the presence and build-up of long chain fatty acids, especially on systems with higher retention times (Dereli et al. 2014). Possibly the utilization of AnMBR on TS preceded by removal of distiller's corn oil may reduce the negative impact of long chain fatty acids hindering AD performance. The combination of thermal processes and AD has been reported as an alternative to the evaporation units of TS, while still producing animal-grade distiller's grains (Wood et al. 2013). The energy efficiency of diverting TS to a hydrothermal carbonization (HTC) and AD processes is reported to be higher than traditional evaporators and AD practices, reducing about 73% of control uses (Wood et al. 2013). AD from TS filtrate consumed over 90% of COD

and converted over 80% COD to $CH_4$, while still providing fatty acid extracts from center point TS hydrochars with 68% yield (Wood et al. 2013). HTC on TS would still provide hydrochars that could undergo post-thermal treatments, possibly enriching their utilization (Wood et al. 2013).

The recyclability of AD-treated TS into fermentation streams presents inhibitory effects for *S. cerevisiae* fermentation performance. The various organic compounds and inorganic macronutrients present in the digestate water (Lin et al. 2015b) may cause potential toxic effects to the yeast metabolism. A study evaluating the effects of ammonium derived from AD in the recyclability of process water, and results show that 200-300 mg $NH^{4+}$-N $L^{-1}$ is a suitable threshold to diminish negative effects on the fermentation process (Wang et al. 2014). Additional negative effects of recycling process water are related to the increase of Maillard reaction products between reduced sugars and ammonium, when digestate water is recycled to the mashing tank, favorably formed during the high mashing temperatures (Wang et al. 2014). Due to the increase in Maillard reaction, less sugar is available to the yeast to ferment, thus, impacting the total carbon amount available to be converted to ethanol. Ammonium nitrogen also favors the glycerol synthesis, which is represented by an undesirable diverted pathway of the ethanol fermentation (Wang et al. 2014). Wood et al. (2013) reported levels of lactic acid below the toxic level for yeast, but inhibitory levels for glycerol and acetic acid from a two-step thermal and AD process. Recycling digestate water, under toxic levels, can represent economic

savings for an ethanol plant not only for decreasing the water input, but also on the replacement of the traditional urea used as nitrogen source to nitrogen sources derived from AD.

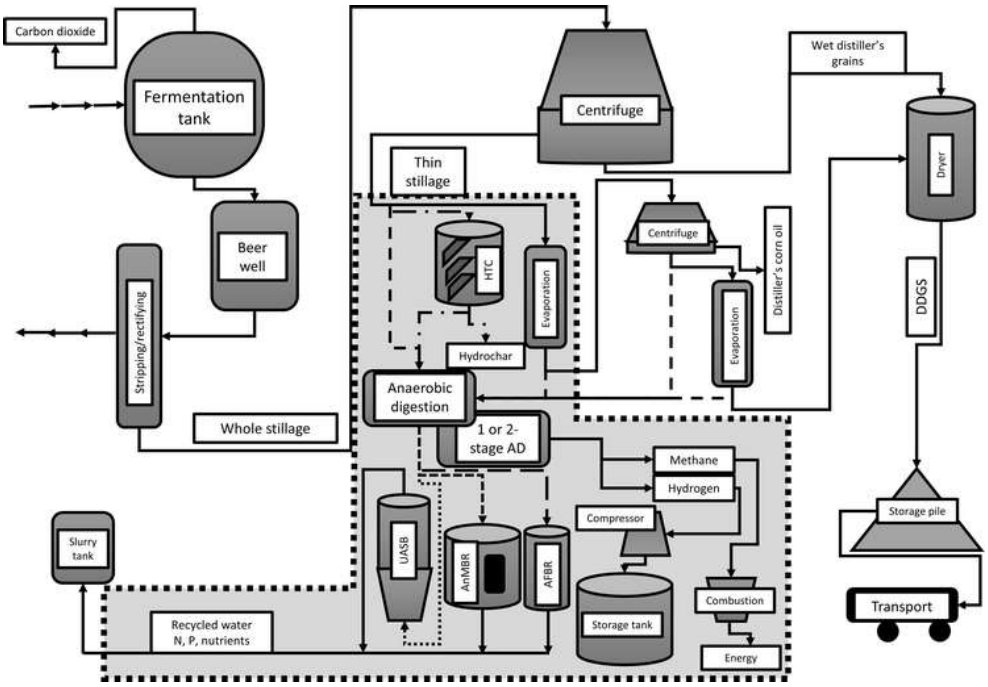


Figure 2.7: Anaerobic digestion practices using thin stillage as substrate

### 2.5.1 Hydrogen production from TS

Anaerobic digestion consists of four main steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. It has been reported that the rate-limiting step for complex organic substrates is hydrolysis, due to the formation of toxic by-products or non-desirable volatile fatty acids (Lay 2000). Methanogenesis, on the other hand, is the rate-limiting step for easily biodegradable substrates. Extensive research has been done on pretreatment methods to accelerate the hydrolysis process to obtain suitable by-products from this step, as well as to

improve the quality of useful compounds, like nitrogen and phosphorus to be recycled.

A significant portion of studies on fermentative $H_2$ production has used pure cultures under sterile conditions. An interesting alternative has been to employ mixed cultures instead of pure culture, dismissing the sterilization needs. Mixed cultures have also been reported to adapt well to the microbial diversity and to handle different substrates, as well as the possibility of conducting continuous processes (Hawkes et al. 2002). $H_2$ production by mixed bacterial culture requires inhibition of the bacteria on methanogenesis (Chen et al. 2008). A strategy often used to contour this step is to inhibit $H_2$ consumption by microorganisms that comprise the anaerobic sludge. Different methods of sludge pretreatment have been reported to avoid the presence of $H_2$ consumers and enrich a mixed culture with $H_2$-producing bacteria. These pretreatments involve mostly acid addition and/or heat (Ariunbaatar et al. 2014).

Bio-hydrogen production from TS has been scarcely reported in the literature. Nasr et al. (2013) developed a comparison between continuous and batch systems to assess bio-hydrogen production from TS. According to their results, yield as high as 19.5 L $H_2$ $L^{-1}$ TS was obtained. They identified few strains responsible for $H_2$ production, which includes *C. acetobutyricum, K. pneumonia, C. butyricum,* and *C. pasteurianum* as the predominant species through a 16S rDNA mapping. The optimum experimental range of substrate to hydrogen ratio was found to be 1-2 g COD substrate $gVSS^{-1}$ seed using conventional anaerobic

digester sludge, while in acclimatized anaerobic digester sludge, the range was found between 3-6 g COD substrate gVSSseed$^{-1}$.

According to Nasr et al. (2012), separating the acidogenic and methanogenic stages in a two-stage anaerobic digestion process has been usually investigated to maximize the acidification process, regardless of the acidification pathways and the hydrogen produced in the first stage. The use of two-stage digestion on TS has led to an increase in the total volatile fatty acids to total chemical oxygen demand from 10% to 56.8% due to acidification process during hydrogen production in the first stage. Comparison of energy outcome from both digestion scenarios revealed an overall increase of 18.5% in energy yield in the two-stage digestion, considering additional energy from hydrogen production.

Anaerobic digestion of TS has been highly considered as a way of generating energy from ethanol coproducts. Even though hydrogen can be produced in many ways, such as electrolysis, photolysis and photo-fermentation, production through anaerobic digestion pathways have become a widely accepted method of biological production, especially when combined with low-value streams. Hydrogen production from TS also helps to resolve the main concern in TS recirculation, i.e., the accumulation of fermentation inhibitors (acetate, glycerol, ethanol, and lactate) in the fermentation tank, since the anaerobic degradation should aid degrading these molecules to volatile products.

### 2.5.2. Water reuse and recyclability of biologically treated TS

A major focus in treating TS is to recycle the water back to the ethanol process, reducing the fresh water requirement for corn-ethanol production. Since TS is

produced at ratios up to 20 fold the ethanol production (Rosentrater and Muthukumarappan 2006), the water footprint of ethanol may be environmentally inhibitive. According to Lim et al. (Lim et al. 2012), the energy demand of the conventional cooking process in ethanol production is equivalent to up to 40% of the fuel value of ethanol produced. Furthermore, a major problem with fermentative ethanol production is the large amounts of organic pollutants generated. As discussed previously, in an industrial distillery, stillage, fermenter and condenser cooling water are, alongside with fermenter wastewater, are the main sources of residual water (Pant and Adholeya 2007). A fungal based purification of thin stillage from dry-grind corn milling was developed using the strains, *Rhizopus oligosporus* and *Mucor indicus* (Van Leeuwen et al. 2015). Despite the ability of the strains to degrade high strength organic wastewater, the fungal biomass has high value as animal feed with high yield. The effluent from the process can be recycled after removing the competitive bacteria by novel disinfection technique (Van Leeuwen et al. 2015).

Several possible ways are attempted to address the issue of attenuating such problem. The design of a zero-discharge non-cooking fermentation system for direct production of ethanol from starch may appear to be the best way to minimize energy costs and the generation of pollutants. The technical limitations involving this approach would be the fact that the two set of enzymes used to degrade starch, 1.4-α-D-glucan glucanohydrolase (α-amylase, EC 3.2.1.1) and 1.4-α-D-glucan glucohydrolase (glucoamylase, EC 3.2.1.3), require moisture on

starch granules (Souza 2010). Starch granules are resistant to amylolytic digestion, thus the reaction kinetics is considerably slow even in favorable conditions, i.e., in submerged systems (Sarikaya and Gürgün 2000). Highly active enzymes with raw starch hydrolytic capabilities were created combining amylases from *Aspergillus kawachi* and glucoamylase from *Aspergillus niger*. The study developed by Shariffa et al. (2009) showed that these have the potential to overcome the physical limitations of the natural set of enzymes which are currently utilized.

The application of enzymes able to hydrolyze granular starch eliminates, or at least reduces, the need of the cost-intensive liquefaction cooking step (Sassner et al. 2008). Such approach would be able to reduce the energy and water inputs per unit of ethanol production. Simultaneous saccharification and fermentation (SSF) techniques have been able to address some of these issues (Galbe et al. 2007). High-density amylases are strongly inhibited by the hydrolysis products, such as glucose, and SSF provides a direct conversion of glucose to ethanol as soon as it is generated. SSF also reduces osmotic stress since the yeast cells are exposed to a relatively lower sugar concentration. Furthermore, SSF allows the reduction in investment and operational costs due to the reduction in the overall fermentation time (Kobayashi et al. 1998).

The use of a zero-discharge fermentation system implies that whole stillage to be sent to a decanter centrifuge. The solids are dried to produce distillers' grains. TS water would be recycled into the liquefaction stage, while the residues from the

bottom of the rectification column and a fraction of solid-rich TS would be directed to the fermentation system (Quintero et al. 2008). The reduction of the amount of fresh water in a desirable system like this implies a considerable improvement over the norm. This technology for alcohol-from-starch has been first described by Nakamura et al. (Nakamura et al. 1997). It has been found that after recycling eight times, the average production yield was still similar to the conventional process (8.8% and 9.0%, respectively). The main drawback is that, the zero-discharge system required a longer fermentation time period, going from 60 h to 90 h. According to the study, the performance of such system is dependent on a number of parameters, such as the composition of the feed and the number of times the liquid is recycled (Nakamura et al. 1997).

Water requirements have been reduced significantly in the ethanol industry (Singh et al. 2001). In 1995, more than 20 gallons of water were needed to process each bushel of corn for ethanol production. In 2001, most plants in the US required less than 7 gallons per bushel of corn. The interest of decreasing water use in the ethanol industry is not solely for environmental purposes, since even for small plants, 300,000 gallons of water are required on a daily basis. Such value can be a large quantity for some municipalities to supply, which may increase significantly transportation costs. Studies in optimizing TS utilization for recycling water within a dry-grind ethanol plant can be one of the most feasible ways to provide zero discharge to plants (Białas et al. 2010).

Use of new alternatives for TS water recycling include the cultivation of fungi (Koza et al. 2017; Rasmussen et al. 2014), algae and fungi combined (Rajendran et al. 2016). Reiterating the summary of section 4, TS is a suitable cultivation medium for fungi. *Rhizopus oligosporus* cultivated in TS decreased 80% of COD, 98% of suspended solids, 100% of glycerol and organic acids, thus, providing water suitable for recycling (Rasmussen et al. 2014). A combination of *Mucor circinelloides* (fungus) and *Chlorella vulgaris* (algae) was able to remove over 55% of phosphorus and 74% of nitrogen on TS, alongside with a COD removal of 62%, decreasing the nutrient discharge to the environment (Rajendran et al. 2016). Utilizing a combination of fungus and algae on nutrient-rich effluents, as TS, may be a possible direction for nutrient recovery into a solid biomass phase, due to the synergistic effect of bioremediation effects of algae (Reis et al. 2014b) and fungi (Rajendran et al. 2016).

**2.6. TS as liquid animal feed**

The chemical composition of ethanol coproducts is directly influenced by the type and cultivar of cereal grain used in the fermentation process, and the efficiency in which starch is converted to ethanol. Thin stillage derived from corn-to-ethanol process usually has less crude protein and neutral detergent fiber levels when compared to TS derived from wheat, barley, rye, and sorghum (Mustafa et al. 2000). Thin stillage, have been recognized as excellent energy and protein sources for several different animals, such as growing and lactating cattle. TS can be used as a partial or complete replacement of water. When TS is fed with poor quality feeds, it acts as an energy and protein supplement. In well balanced

diets, however, TS improves feed efficiency by reducing dry matter intake. Additional consideration should be given to lysine supplementation, since it tends to be deficient in corn TS.

A study conducted by Han and Liu (2010) evaluated the amino acid profile and nitrogen compounds in ethanol coproducts. Their samples consisted on average 70.2% starch, 7.65% protein, 3.26% oil, and 1.29% oil. According to their findings, after fermentation, starch content decreased to about 6.0%, while protein, oil, and ash contents increased over 3-fold, and amino acids increased at least 2 fold. Table 2.2 summarizes amino acid profile for corn TS samples, based on Wu et al. (1989) and Han and Liu (2010). Both characterization results differ significantly and no direct conclusion can be made on whether TS carries an increase in concentration of a specific amino acid.

In TS, more than 60% crude protein is bound to the neutral detergent fibers (NDF) while most of soluble protein is found as non-protein nitrogen. Mustafa et al. (2000) evaluated the high levels of neutral detergent insoluble protein in TS and concluded that these levels explain the relatively high NDF in TS. Some of the reported starch values for corn TS are exceptionally high (Larson et al. 1993), most likely due to the differences in distillation methods. The amino acid composition of a given cereal TS is close to that of the original cereal grain. It has been reported that glutamic acid is the most abundant amino acid in TS, followed by leucine in corn. It has also been reported that TS derived from barley has a superior amino acid composition relative to wheat and corn (Mustafa et al. 1999).

A study done by Larson et al. (1993) evaluated the use of wet distillers' coproducts, such as wet distillers grains and TS in the feed composition of finishing ruminants. They conclude that TS as protein and energy source enables rapid and concentrated dispersion, and when fed up to 40% of the diet on a dry basis, their results contained 2.53 Mcal of net energy for gain per kg for yearlings and 1.96 Mcal for calves, which is 63% and 26% greater respectively, when compared to corn cereal (table 3).

Table 3. Corn grain and TS amino acid composition (% of protein)

| **Amino acid** | **Grain*** | **TS*** | **Grain**** | **TS**** |
|---|---|---|---|---|
| Arginine | 5.8 | 5.6 | 3.55 | 6.11 |
| Histidine | 2.9 | 2.7 | 3.13 | 3.84 |
| Isoleucine | 3.2 | 4.0 | 3.61 | 2.84 |
| Leucine | 11.9 | 12.1 | 12.23 | 7.95 |
| Lysine | 3.1 | 3.6 | 3.19 | 4.76 |
| Methionine | 1.3 | 1.8 | 3.19 | 3.55 |
| Phenylalanine | 4.7 | 5.2 | 6.49 | 4.47 |
| Valine | 4.8 | 5.5 | 7.5 | 6.11 |
| Non Essential | | | | |
| Alanine | 6.9 | 7.2 | 6.56 | 7.60 |
| Aspartate | 6.2 | 7.5 | 5.91 | 7.46 |
| Glutamate | 18 | 16.9 | 17.73 | 19.03 |
| Glycine | 3.7 | 4.2 | 3.43 | 5.47 |

| | | | | |
|---|---|---|---|---|
| Proline | 8.8 | 8.2 | 6.67 | 4.97 |
| Serine | 4.6 | 5.1 | 5.02 | 5.11 |

* Adapted from (Wu et al., 1989)

** Adapted from (Han & Liu, 2010)

Hanke et al. (1983) evaluated TS as a replacement for drinking water in three feedlot trials to steers. When these animals were fed with TS, the mass gained was 5.7% faster, consumed 5.8% less dry matter, and required 11% less dry matter per kg of gain than steers receiving water. It has been suggested by the authors that enhanced performance may have been due to high levels of trace elements in TS or may be due to the change in ruminal digestion of carbohydrate and protein. A posterior study developed by Ham et al. (1994) indicated that a portion of TS may have bypassed the rumen. According to their assumptions, 50% of TS bypassed the rumen in growing cattle, and 0.8 kg of DM from TS entered the small intestine for enzymatic digestion. These authors believe that fermentation of starch and protein results in lost energy efficiency due to the heat of fermentation (Faichney et al. 1981). Rust (1991) reported that decanted TS, with 7.56% of dry matter, provided 4.68 Mcal $kg^{-1}$ when replacing drinking water of steers, fed up to a 90% concentrate diet. A study with steers being feed different concentrations of condensed solubles, i.e., TS with lower moisture content, concluded that levels of 10% of inclusion in the diet do not influence negatively growth performance or weight gain efficiency (Weiss et al. 2016). The

feeding inclusion of TS in animal feed under a threshold, which varies by animal, does not alter live animal performance and carcass characteristics (Weiss et al. 2016).

## 2.7. Conclusions

Innovative ways of utilizing TS from corn ethanol processing for generating new products and processes could benefit the economics and ecology of the corn ethanol industry. The physio-chemical treatment or biological utilization of TS could result in a new high value product which could transform the future of corn ethanol industry. The research reports show that the TS was suitable as the culture medium for the microbial production of many bio-products.

The U.S. ethanol industry is entering a new era where ethanol plants are evolving into biorefineries. This evolution is driven by the sometimes narrow ethanol profit margins and increased dependence on revenues from coproducts. Due to market size, DDGS are expected to be the leading coproduct in commercial volume. It is important to note that several of the proposed modifications presented in this review can directly impact the quality of distiller's grains as feeding material. Technologies such as phosphorus removal, backset of TS and addition of external enzymes can lead to higher digestibility rates and degradation of anti-nutritional components present in TS, such as non-starch polysaccharides. Overall, since a large number of ethanol plants in the United States is owned by local farmers, it is expected that novel and breakthrough technologies can provide higher payback rates per corn processed, increasing competitiveness and enhancing the social contribution of ethanol production.

# CHAPTER 3. RECENT UPGRADES IN DRY-GRIND ETHANOL PLANTS IMPACT PHOSPHORUS DISTRIBUTION: A SURVEY AND MASS BALANCE ANALYSIS

## Outline

Recent upgrades in the corn-to-ethanol industry directly impact the phosphorus characterization in the downstream processing. The most signifficant change regarding phosphorus distribution in ultimetaly animal feed is the addition of phytases to upstream and fermentation processes – partially degrading organic phosphorus to reactive-phosphorus. The addition of phytases have consequences on the amount of phosphorus that is recycled in anaerobic digestion operations in ethanol plants, recycling 100% less when compared to plants that do not add phytase to their streams on a whole stillage basis. This study evaluated downstream processing samples from four large-scale operations in the Midwest of the United States, and provides a compositional analysis of ethanol coproducts on regards to inositol phosphate distribution, phosphorus mass balance and impacts of phytase addition on amount of phosphorus recycled via anaerobic digestion, on oil extraction, and on the phosphorus concentration and distribution in distiller's grains.

### 3.1.1. Introduction: corn-to-ethanol production

Corn (*Zea mays*) is the most employed feedstock in the whole world to produce starch for the food industry or for ethanol production(Mumm et al. 2014). Corn is also the most cropped grain globally, followed by wheat and rice, and the United States is the global leader in production numbers, having over 83 million acres of

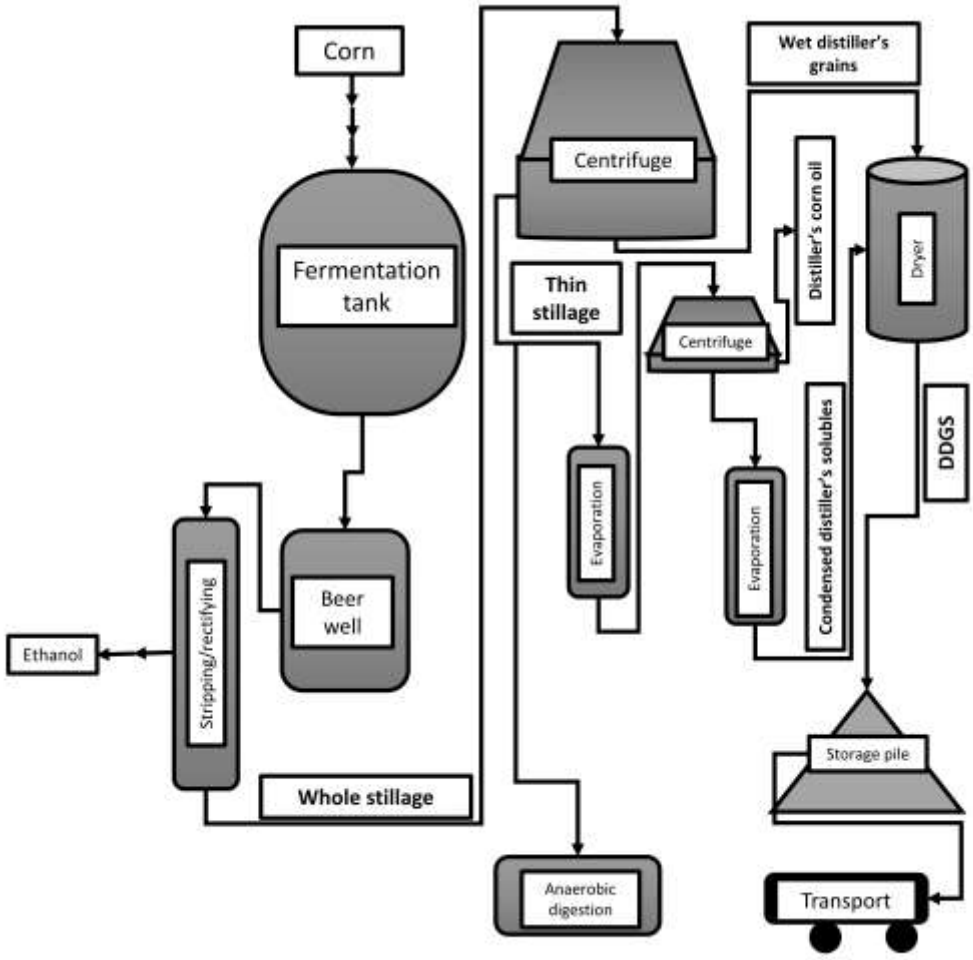
harvested corn in 2014 (Sakamoto et al. 2014).Starch is the corn component that is used as feedstock for ethanol production. Among the technologies for converting corn-based starch to ethanol, two processing methods correspond to the production unanimity. One which is based on the separation of starch from all other components of the corn kernel, called wet-milling process, and one, entitled dry-grind, which does not separate starch from the kernel (Cardona and Sánchez 2007).

Nowadays, most industries in the United States utilize the dry-grind process, which approximately produces roughly 90% of corn ethanol (Shurson 2016), because of numerous advantages, especially related to the coproducts generated. During the dry-grind corn processing, the whole kernel enters the ethanol production line, and consequently, all the parts of the corn kernel are processed simultaneously with the starch material (Kwiatkowski et al. 2006). The non-utilized parts of kernel are usually accumulated on the bottoms of the first distillation column, and these are concentrated to form a product utilized as animal feed (Liu and Han 2011).

The stillage recovered from the concentration column, produced generally at a 3:1 ratio (3 parts of stillage for 1 part of ethanol) is sent to a centrifuge (Kwiatkowski et al. 2006). The solids, wet distiller's grains (WDG), are produced a co-product used for animal feed, rich in nutrients, nutrients, and vitamins (Kim et al. 2008b). The liquid fraction, thin stillage, is sent to an evaporator, usually a double-effect type of evaporator, and the obtained syrup, known as Condensed

Distiller's Solubles (CDS) is combined with wet distiller's grains (WDG) and the overall mixture is dried. The condensed water is recirculated to the liquefaction stage of the evaporators, while the fractions present at the rectification column part are recycled to the saccharification step (Cardona and Sánchez 2007).

Figure 3.1.: Simplified dry-grind downstream processing




### 3.1.2. Introduction: Phosphorus in ethanol coproducts

The class of organic compounds known as inositol phosphates comprehend a group of phosphorus chemicals found in the natural environment, common in eukaryotic organisms, especially in plants, where they constitute most of the

phosphorus in the seeds (Ravindran et al. 1994). The abundance of these inositol phosphates in nature means that they are widespread in the environmental and ecological sciences, and their impact is significant on most processes they are present. It has been previously stated that the coproducts generated at dry-grind corn ethanol facilities are used as animal feed, especially the DDGS (Noureddini and Dang 2009). However, it is known that monogastric animals have low digestibility of phytate, i.e., the salt form of myo-inositol hexakisphosphate, and also the most abundant inositol phosphate in cereal grains (De Boland et al. 1975). Inositol hexakisphosphate excreted by monogastric animals plays an important role in linking the phosphorus present in soil to plants(George et al. 2005). However, due to the excessive amounts of P in DDGS, i.e. over 10 g $kg^{-1}$ (Spiehs et al. 2002), about 70% of the Total-P intake is lost in excreta(Ammerman et al. 1995), which is widely used as fertilizer.

As manure is applied as fertilizer, phosphorus compounds tend to accumulate in the soil and there are eventual runoffs to waterbodies, causing, among other problems, eutrophication (Noureddini et al. 2009). In order to address this issue, several techniques or strategies have been developed to improve animal digestibility of phytate. One of the strategies is the use of ‘low-phytate’ grains, which are mutants selected for the low-concentration of inositol phosphate in their seed (Sokrab et al. 2014). Other is based on the development of transgenic animals able to produce phytase, an enzyme that degraded phytate, which is not found naturally in the guts of monogastric animals (Golovan et al. 2001). The

third, and by far, the most successful strategy, is the supplementation of animal diets with microbial phytase (Dersjant-Li et al. 2015). This practice is considered standard in most large-scale animal feeding operations, and it has proved to be effective in reducing phosphorus excretion in manure, and as a beneficial side-effect of additional metal supplementation provided by the previously complexed metals bound with phytate (Ekholm et al. 2003).

It is also known that a more novel technology utilizes microbial phytase in the corn milling process, having phosphorus liberated and utilized throughout the fermentation (Khullar et al. 2011; Noureddini and Dang 2009). This has proved to even increase fermentation yields bringing extra revenues to the plants (Khullar et al. 2011). There are companies, on the other hand, that still do not apply this kind of technology, and have relatively phytate-rich coproducts, which could potentially be recovered and processed (He et al. 2017b). The purpose of this chapter is to analyze coproducts from four companies from the Corn Belt region in the United States, based in Minnesota, Iowa, Wisconsin, and Illinois, on regards to the phytate availability, Total-Phosphorus, as well as reactive-Phosphorus, i.e., free orthophosphate. The analysis presented on this article addresses the environmental impacts of current recent upgrades of the dry-grind ethanol industry on the phosphorus discharge in animal feed.

## 3.2. Materials and Methods

### 3.2.1. Sample collection, storage and chemicals

Samples from 4 dry-grind corn ethanol companies in the Midwest were collected and analyzed. The sample codes used in these results are the following, using

the technical names provided by each company:

Company 1, based in Illinois: Whole Stillage (WS-1), Thin Stillage before Oil Extraction (BOTS-1), Thin Stillage after Oil Extraction (AOTS-1), Condensed istiller's syrup (CDS-1), Wet distiller's grains (WDGS-1), and Dried Distiller's Grains with Solubles (DDGS-1).

Company 2, based in Minnesota: Whole Stillage (WS-2), Thin Stillage (TS-2), Syrup (CDS-2), De-oiled Syrup (DCDS-2), Wet Distiller's grains with solubles (WDGS-2), and Dried Distiller's Grains with Solubles (DDGS-2).

Company 3, based in Iowa: Whole Stillage (WS-3), Thin Stillage (TS-3), Thin Stillage before Oil Extraction (BOTS-3), Thin Stillage after Oil Extraction (AOTS-3), Syrup (CDS-3), Wet distiller's grains (WDG-3), Wet distiller's grains with solubles (WDGS-3) and Dried Distiller's Grains with Solubles (DDGS-3).

Company 4, based in Iowa: Whole Stillage (WS-4), Thin Stillage before Oil Extraction (BOTS-4), Thin Stillage after Oil Extraction (AOTS-4), Wet distiller's grains with Solubles (WDGS-4), and Dried Distiller's Grains with Solubles (DDGS-4).

All samples were kept in the refrigerator at -20 $^{o}$C for storage until analysis. P kits (TNT 845/Hach, Loveland, CO) were used to measure the P. All chemicals used were in analytical grade.

### 3.2.2. Sample analysis and preparation

All samples were analyzed to quantify contents of moisture, total-P and phytate-P. The data for each stream was replicated three times for three independent samples. Moisture calculation was based on loss-on-drying AOAC 935.29

method (Thiex 2008). Total-Phosphorus was based on AOAC 942.05 method (Thiex et al. 2012), in which samples were converted to ash, followed by $HNO_3$ 7 mol $L^{-1}$ addition to dissolve P present in ash, and measured using an adapted Molybdenum blue assay(Murphy and Riley 1962).

Phytate-P was quantified using an indirect method based on the difference of total and reactive-P. In this method, Total-P was precipitated with calcium chloride, followed by HCl digestion (He et al. 2017a). Inositol phosphate distribution was performed by AB Vista (Marlborough, United Kingdom) with $^{31}P$ NMR(Frølich et al. 1986).

Phosphorus flow

Phosphorus flow from coproducts was determined using mass, solids, and Total-P balance for each of the stillage steps. The phosphorus flow was based on a mass balance model (Winsness and Barlage 2008),which include removal of oil from concentrated thin stillage, as this was present in three out of the four companies. Equations 1, 2, and 3 were used to characterize the mass balance on each step.

$$\sum_{i=1}^{n} \boldsymbol{m_i} = \sum_{j=1}^{k} \boldsymbol{m_j} \quad (1)$$

$$\sum_{i=1}^{n} (\boldsymbol{m_i S_i}) = \sum_{j=1}^{k} (\boldsymbol{m_j S_j}) \quad (2)$$

$$\sum_{i=1}^{n} (\boldsymbol{m_i P_i}) = \sum_{j=1}^{k} (\boldsymbol{m_j P_j}) \quad (3)$$

Where $m_i$ = total mass input for input i (g), $m_j$ = total mass output to a process for output j (g), $S_i$, $S_j$ = solids content in the inputs and outputs (g $g^{-1}$ sample – wet basis), and $P_i$, $P_j$ = Total-P content of the inputs and outputs (mg P $g^{-1}$ sample –

dry basis).

### 3.2.3. Model assumptions

The assumptions used to build the mass balance model are based on previous characterizations of ethanol coproducts.

(i) Production volume of ethanol coproducts: the starting point for volumetric production of ethanol coproducts was the amount of TS produced by company 3: 1013760 gallons of TS per day. Company 3 is a plant producing 100 million gallons of ethanol per year, operating near its full capacity, which does not differ much of most ethanol facilities in the U.S. in the recent years (Ramchandran et al. 2015).

(ii) Volumetric flow to mass flow conversion: TS has specific gravity levels reported within the range of 1.01 to 1.03 (Ratanapariyanuch et al. 2011). Laboratory measurements of TS-3 sample used in this study as 1.01 ± 0.02.

(iii) (Rasmussen et al. 2014): The amount of TS sent to anaerobic digestion operations before, after, or during evaporation units ranges from 15% (Kim et al. 2008) to 58% (Rasmussen et al. 2014). Company 3 recycles an average of 50% of produced TS in anaerobic digesters. The backset ratio (volume of TS recycled after the first evaporation unit/total volume of TS processed after the first evaporator) was defined as 0.5 in this study. It was assumed that all phosphorus compounds are converted to simple orthophosphate forms after anaerobic digestion (Alkan-Ozkaynak and Karthikeyan 2011), and are sent to either fermentation (Kwiatkowski et al. 2006) or the slurry tank (Han and Liu 2010). It was also assumed that the orthophosphate generated from the anaerobic

digestion of TS is recycled in order to reduce exogenous phosphorus supplementation to the yeast fermenting the saccharified mash (Kim et al. 2008a).

(iv) Mass constraints and oil extraction: Adapting the modelling developed by Kwiatkowski et al. (2006), it was assumed that the drying of TS through the first to second evaporator did not affect the dry weight flow, i.e., for model building purposes, it considered that the total solids present in TS did not vary in between evaporation units. On a similar consideration, it was assumed that the drying of WDGS did not alter the dry weight of DDGS – thus, the dry weight flow of WDGS is the same than that of DDGS. Oil content of thin stillage was considered 1.3%(Reis et al. 2017), and P content of oil was 0.04%(Bruinsma et al. 2016).

### 3.3. Results and discussion

#### 3.3.1. Composition of corn ethanol coproducts

The composition of the coproducts studied showed a wide variance among the plants. Phosphorus is a key issue to consider in these products, especially on those which are applied to animal feed, i.e., DDGS. Physical composition did not vary significantly among the same category of product (e.g. WS) for different plants, except for TS.

TS is a broad term for a characteristic mixture present from the solid/liquid separation in Whole Stillage up to the evaporation to a syrup material, CDS(Reis et al. 2017). Plant 3 was the only one that provided the fractions of TS just after centrifugation, and fractions that have been through a degree of evaporation, both prior and past the additional centrifugation for distiller's corn oil extraction.

After oil extraction, moisture contents were within the range of 68.87% to 79.10%, considerably close to that of CDS (65.67% to 72.29%). There is not sufficient data available on the scientific literature to contrast and compare the moisture content levels of the intermediates of thin stillage. However, as shown on table 1, moisture content levels for CDS are similar for all samples. Table 1 compares samples with other available characterizations: Alkan-Ozkaynak and Karthikeyan (2012), the three samples analyzed by Liu and Han (2011), and Noureddini et al. (2009), respectively represented at table 1 as samples 1 to 3. It can be hypothesized that the 4 lot of samples are from companies that apply different fermentation and downstream conditions in their processes, mostly due to the difference in phytate-P percentage.

Due to the P profiling, it can be assumed that 2 out of the 4 companies add phytase to their system, due to their significant increase in reactive-Phosphorus and decrease in higher forms of inositol phosphates(Khullar et al. 2011; Noureddini and Dang 2009; Noureddini and Dang 2010). It has been claimed by Liu and Han (2011) that P is among the most important minerals to be considered in ethanol coproducts. They evaluated at which steps in the dry-grind ethanol process, P went through significant changes in form. The current study confirms the results presented by Liu and Han (2011), not only differentiating the P forms in total-P, reactive-P, and phytate-P, but also by differentiating phytate as the fractions of inositol phosphates ($IP_2$, $IP_3$, $IP_4$, $IP_5$, and $IP_6$).

Table 3.1.: Characterization of ethanol coproducts on regards to Moisture Content (MC), Phytate-P (PP), Reactive-P (RP), and Total-P (TP) for plants 1-4. NR: non-reported

| Sample | Source | Moisture content (%) | PP (mg $g^{-1}$) | RP (mg $g^{-1}$) | TP (mg $g^{-1}$) |
|---|---|---|---|---|---|
| WS | Plant 1 | 87.51±0.11 | 1.98±0.40 | 6.72±0.15 | 11.76±0.35 |
| | Plant 2 | 86.32±1.32 | 2.04±0.04 | 3.60±0.22 | 9.09±0.10 |
| | Plant 3 | 87.94±0.15 | 4.81±0.01 | 3.18±0.18 | 12.02±0.60 |
| | Plant 4 | 87.57±1.98 | 5.27±0.23 | 3.29±0.33 | 11.33±0.71 |
| | Alkan-Ozkaynak et al. | 87.7 | NR | NR | 11.0±0.04 |
| | Liu and Han - Sample 1 | NR | 5.24 | 2.95 | 11.11 |
| | Liu and Han - Sample 2 | NR | 5.5 | 2.74 | 11.52 |
| | Liu and Han - Sample 3 | NR | 6.37 | 3.28 | 12.99 |
| | Noureddini et al. | 82.7±1.5 | NR | 5.2±0.7 | 8.8±0.6 |
| WDG | Plant 3 | 62.57±0.52 | 2.01 ± 0.12 | 0.81±0.05 | 5.37±0.40 |
| | Alkan-Ozkaynak et al. | 66.1 | NR | NR | 4.62±0.18 |
| | Liu and Han - Sample 1 | NR | 2.32 | 1.07 | 4.55 |
| | Liu and Han - Sample 2 | NR | 2.59 | 0.91 | 5.23 |
| | Liu and Han - Sample 3 | NR | 2.22 | 0.78 | 4.34 |
| TS | Plant 2 | 93.81±0.67 | 5.58±0.04 | 9.68±0.20 | 21.70±0.76 |
| | Plant 3 | 95.71±0.13 | 10.11±0.66 | 7.27±0.48 | 23.57±1.12 |
| | Alkan-Ozkaynak et al. | 93.1 | 9.66 | 7.9 | 19.4±0.01 |
| | Liu and Han - Sample 1 | NR | 9.2 | 5.63 | 18.69 |
| | Liu and Han - Sample 2 | NR | 9.4 | 6.62 | 19.01 |
| | Liu and Han - Sample 3 | NR | 11.01 | 6.62 | 21.65 |
| BOTS | Plant 1 | 92.88±0.36 | 2.24±0.20 | 14.73±0.55 | 20.33±0.26 |

| | | | | | |
|---|---|---|---|---|---|
| | Plant 3 | 69.91±0.38 | 8.24±0.14 | 6.66±0.20 | 20.90±0.87 |
| | Plant 4 | 91.94±0.06 | 4.09±0.32 | 3.27±0.30 | 16.56±0.68 |
| AOTS | Plant 1 | 68.87±0.04 | 2.48±0.40 | 15.45±0.18 | 20.32±0.26 |
| | Plant 3 | 73.87±0.17 | 9.39±0.53 | 7.59±0.30 | 21.23±0.60 |
| | Plant 4 | 79.10±0.08 | 8.62±0.15 | 6.78±0.16 | 20.13±0.37 |
| CDS | Plant 1 | 68.43±0.06 | 1.78±0.13 | 14.13±0.02 | 22.69±0.34 |
| | Plant 2 | 72.29±0.01 | 2.82±0.29 | 10.07±0.14 | 17.02±0.41 |
| | Plant 3 | 70.48±0.22 | 9.41±0.33 | 7.68±0.51 | 23.77±0.23 |
| | Plant 4 | 65.67±0.42 | 6.73±0.29 | 5.72±0.15 | 18.69±0.65 |
| | Alkan-Ozkaynak et al. | 61.2 | NR | NR | 18.8±0.10 |
| | Liu and Han - Sample 1 | NR | 7.67 | 6.08 | 17.01 |
| | Liu and Han - Sample 2 | NR | 8.23 | 6.82 | 18.5 |
| | Liu and Han - Sample 3 | NR | 9.96 | 7.42 | 20.76 |
| | Noureddini et al. | 64.9±0.7 | NR | NR | 13.4±0.5 |
| DCDS | Plant 2 | 75.63±0.57 | 3.64±0.21 | 11.71±0.09 | 20.21±0.35 |
| WDGS | Plant 1 | 51.89±0.06 | 0.61±0.27 | 6.48±0.41 | 10.25±0.22 |
| | Plant 2 | 67.11±0.06 | 1.59±0.10 | 1.57±0.14 | 5.28±0.08 |
| | Plant 3 | 49.75±0.40 | 3.27±0.21 | 2.44±0.08 | 10.21±0.53 |
| | Plant 4 | 62.42±0.37 | 3.59±0.20 | 3.44±0.11 | 10.66±0.03 |
| | Alkan-Ozkaynak et al. | 66.1 | NR | NR | 4.62±0.18 |
| | Noureddini et al. | 66.8±1.2 | NR | NR | 8.6±0.4 |
| DDGS | Plant 1 | 14.06±0.47 | 0.88±0.06 | 5.92±0.14 | 8.95±0.34 |
| | Plant 2 | 13.03±0.42 | 1.79±0.25 | 4.12±0.51 | 7.99±0.36 |
| | Plant 3 | 14.33±0.39 | 4.50±0.15 | 3.48±0.12 | 10.59±0.20 |
| | Plant 4 | 12.05±0.25 | 2.36±0.15 | 4.12±0.04 | 9.45±0.28 |

| | | | | |
|---|---|---|---|---|
| Alkan-Ozkaynak et al. | 9.6 | 4.04 | 3.42 | 7.46 |
| Liu and Han - Sample 1 | NR | 3.76 | 3.04 | 8.56 |
| Liu and Han - Sample 2 | NR | 3.79 | 2.56 | 8.35 |
| Liu and Han - Sample 3 | NR | 3.88 | 2.64 | 9.28 |
| Noureddini et al. | 5.7±0.8 | NR | NR | 5.2±0.2 |

### 3.3.2. Inositol-Phosphate distribution among different plants

Two main factors are evaluated on the IP distribution: (i) heat treatment, and (ii) solid/liquid partitioning. References to date describe the presence and flow of phytate-P as only the P fraction of the IP range of molecules(Alkan-Ozkaynak and Karthikeyan 2012; Liu and Han 2011). A $^{31}$P-NMR analysis carried out on all the samples characterized the different IP profiles among coproducts of all companies. Results are found on table 3.2 on an nmol $g^{-1}$ basis and as relative concentrations.

(i) Heat treatment: Oil is removed from Thin Stillage usually after the heat treatment applied to the evaporators, i.e., in the transition from Thin Stillage to CDS. It has been observed for companies 1 and 3, which still have at least 40% of phytate-P present as $IP_6$ in all coproducts, that the heat is likely to degrade $IP_6$ to lower fractions of inositol phosphates. Both for companies 1 and 3, $IP_6$ is present at higher percentages among the inositol phosphates (40.2% and 38.3%, respectively) at Thin Stillage before oil extraction than after oil extraction (34.5% and 37.2%, respectively). Due to the pH of Thin Stillage being acidic (4.4), the combination of heat and acidity can yield partial degradation of $IP_6$ to lower fractions (Phillippy et al. 1987). On regards to company 2, which has most of phytate-P present as $IP_2$ and $IP_3$ on Thin Stillage before oil extraction (75.5% and 24.4%, respectively), thin stillage after oil extraction, i.e., after the treatment by multiple-effect evaporators, their relative concentrations indicate an increase in $IP_2$ and decrease in $IP_3$ (91.0% and 3.7%, respectively).

(ii) Solid/Liquid partitioning: the separation of Whole Stillage to Thin Stillage and

Wet Distiller's grains is processed using a centrifugation unit. WS for companies 1 and 3, i.e., those that are assumed to have little to no exogenous phytase addition to their system, present a higher concentration of $IP_6$ than TS (46.0% and 45.9%, respectively for Whole Stillage, and 40.3% and 38.2%, respectively for TS). Based on solubility studies of inositol phosphates(Cigala et al. 2010; Crea et al. 2008), it is hypothesized that such reduction in relative concentration may be due to partial sorption of phytate-P to the solid particles as WDG, or even present within the solid structure of WDG particles. Inositol phosphates act as strong ligands because of their high anionic charge, and their potential for complexing polyvalent cations have been documented in biological applications(Haydon and Cobbett 2007). The presence of inositol phosphates in WDG is potentially linked to its binding to protein or complex cationic substrates (Turner et al. 2002). It is known that the greater the degree of phosphate substitution on the inositol molecule, the lower the solubility in polar solvents. Inositol-phosphate profiling in WDGS should be the added contributions of the condensed solubles and those of WDG. WDGS presents relative concentrations of $IP_6$ at 36.8% and 35.8% for companies 1 and 3, respectively, while condensed solubles have relative concentrations of $IP_6$ as 32.5% (company 1) and 34.9% (company 3). The increase in relative concentration when combining the liquid (CDS) and solid (WDG) fractions indicate a possible higher relative concentration of $IP_6$ in WDG. The most common phytases used in the industry include Natuphos ® (BASF Animal Nutrition), Ronozyme ® (DSM), and QuantumBlue ®

phytase (AB Vista). Two main categories of phytase enzymes are produced and traded, and include 3-phytase (EC 3.1.3.8), and 6-phytase (EC 3.1.3.26), which names derive from their action site on the phosphate bond on myo- $IP_6$ structure – phosphate bond 3 and 6, respectively (Turner et al. 2002). Commercial phytases have been reported to have enzyme impurities, such as acid phosphatases (Turner et al. 2002). Commercial phytases, thus, usually are able to degrade to a certain degree other orthophosphate monoesters, such as sugar phosphates and mononucleotides (Turner and McKelvie 2002).

Table 3.2.: IP distribution of ethanol coproducts

| Sample | Concentration (nmol $g^{-1}$) | | | | | |
|---|---|---|---|---|---|---|
| | $IP_2$ | $IP_3$ | $IP_4$ | $IP_5$ | $IP_6$ | Inositol |
| AOTS-1 | 2439 | 3590 | 1010 | 1796 | 4660 | 790 |
| BOTS-1 | 663 | 1639 | 369 | 230 | 1952 | 323 |
| CDS-1 | 5528 | 5388 | 1696 | 2750 | 7388 | 174 |
| WS-1 | 511 | 1206 | 439 | 292 | 2081 | 323 |
| DDGS-1 | 7366 | 6758 | 1900 | 3343 | 11279 | 694 |
| WDGS-1 | 903 | 1184 | 538 | 640 | 1475 | 17 |
| AOTS-2 | 6623 | 272 | 292 | 0 | 88 | 3591 |
| BOTS-2 | 1285 | 416 | 0 | 0 | 0 | 466 |
| CDS-2 | 5969 | 192 | 238 | 0 | 0 | 2763 |
| WS-2 | 1215 | 296 | 91 | 0 | 0 | 538 |

| | | | | | | |
|---|---|---|---|---|---|---|
| DDGS-2 | 5966 | 273 | 322 | 181 | 0 | 4080 |
| WDGS-2 | 2797 | 179 | 379 | 0 | 0 | 6890 |
| AOTS-3 | 3927 | 6222 | 1354 | 2285 | 8543 | 819 |
| BOTS-3 | 4105 | 5405 | 1294 | 2005 | 7608 | 678 |
| CDS-3 | 5451 | 6626 | 1515 | 2719 | 8778 | 865 |
| WS-3 | 687 | 1292 | 480 | 73 | 2149 | 119 |
| DDGS-3 | 5814 | 6138 | 2168 | 3474 | 9797 | 1312 |
| WDGS-3 | 1986 | 2548 | 1947 | 2052 | 2679 | 1510 |
| WS-4 | 764 | 1020 | 205 | 434 | 855 | 506 |
| TS-4 | 639 | 993 | 126 | 238 | 493 | 640 |
| CDS-4 | 2415 | 3140 | 508 | 836 | 499 | 3777 |
| DCDS-4 | 3355 | 3142 | 417 | 726 | 472 | 3874 |
| DDGS-4 | 5515 | 5139 | 1487 | 1538 | 3119 | 3801 |
| WDGS-4 | 403 | 896 | 269 | 0 | 1150 | 571 |

The concentration of $IP_{4-6}$ being lower than $IP_{2-3}$ can be explained by the kinetics of dephosphorylation by phytases. Kemme et al. (1999) described the kinetics of $IP_{4-6}$ degradation to be significantly greater than the counterpart of $IP_{2-3}$. The increase in concentration in $IP_2$ may be explained by the high activation energy

required to degrade $IP_2$ to $IP_1$ and inositol (Shun et al. 1994). The increase in inositol concentration in the samples from plants 2 and 4 can be linked not the addition of phytase enzyme *per se*, but to the potential impurities of acid phosphomonoesterase enzymes (EC 3.1.3.1), which can dephosphorylate all inositol phosphates, except for $IP_6$ (Marko-Varga and Gorton 1990).

### 3.3.3. Phosphorus mass balance for a 100-million gallon plant

Plant 3 was selected as the model for building the complete phosphorus mass balance due to the most complete sampling profile. Though it is known fluctuations in the flow during the fermentation batch times are normal, since fermentation processes occur in batch, and downstream processing relies on the fermentation times. Kwiatkowski et al (2006) reported an industrial fermentation time of 68 hours; close to the 60 hours performed for plant 3, in its 2 parallel fermenters.

Due to simplification, it is assumed that the downstream process occurs steadily for 24 hours a day. The purpose of this article is not to describe stochastic variations in the downstream processing, but to evaluate the phosphorus flow within different fractions of the ethanol coproducts.

Whole stillage is processed at a rate of 878467 kg per hour, corresponding to 1484 kg of Total-P per hour. Data from company 3 indicates phytate levels correspondent to those of normal degradation capacity by yeast (Han and Liu 2010).

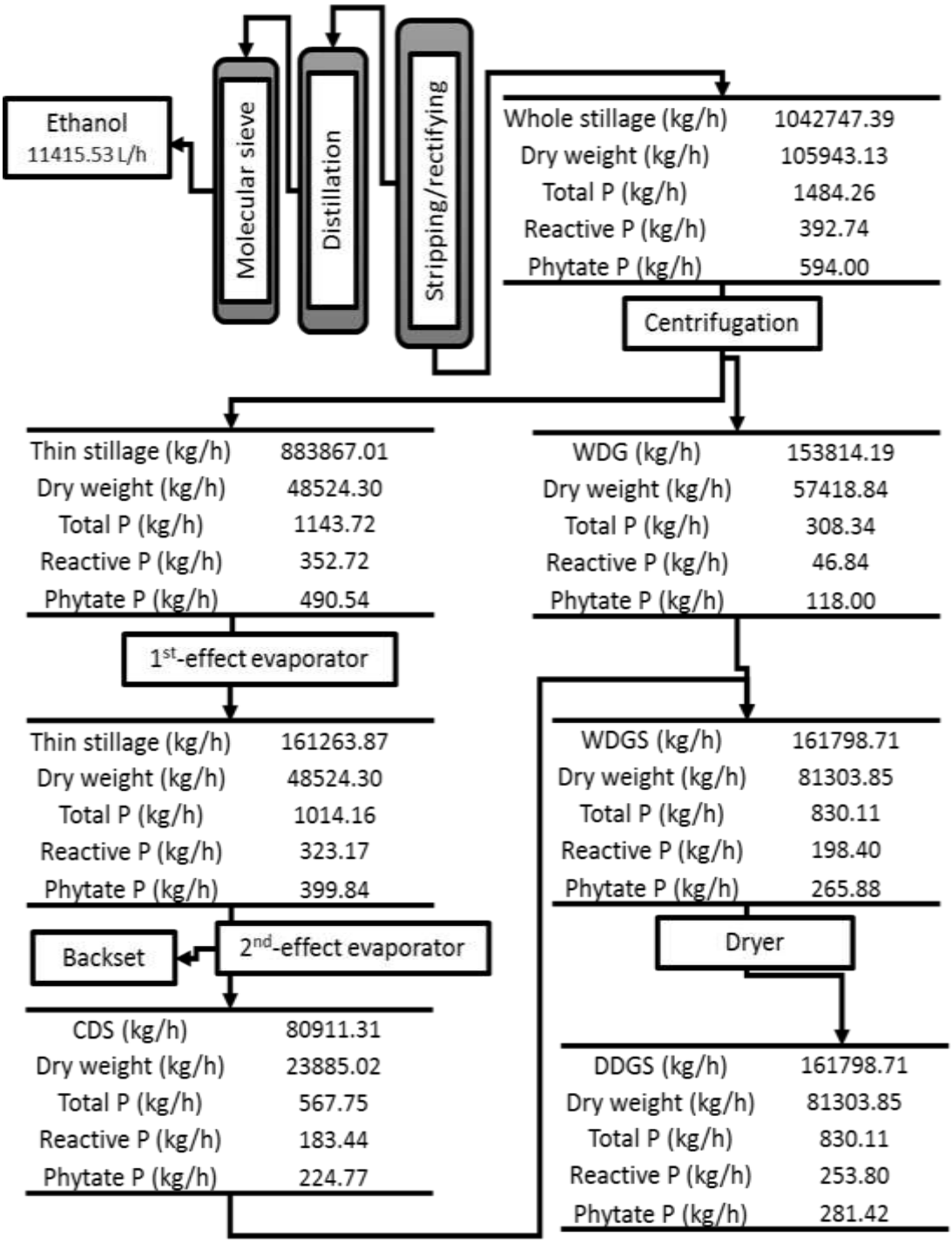



Figure 3.2.: Mass balance for a 100-million year dry-grind corn-to-ethanol plant

594 kg of phytate-P are processed on an hourly basis from whole stillage, which

are divided into a liquid-rich and a solid-rich fraction: TS and WDG, respectively. WDG accounts for about 17% of the total mass of WDG, and about 20.7% of the Total-P contained in WS. On a solid basis, WDG accounts for over 54% of the total mass of the dry mass of WS, containing most of undissolved yeast debris, and corn solid residues with particle diameter greater than 2 mm. TS is processed at a rate of almost 700 metric tons per hour, which is correspondent to about 48 metric tons of solids on an hourly basis. Most of the Total-P from WS is sent to TS, approximately 80%, and over 82% of the Total -P present in WS is processed within the TS fraction. TS has an approximate residence time of 7 hours(Kwiatkowski et al. 2006), and Phytate-P flow in TS is about 490 kg per hour, which does not degrade significantly during the heating evaporation units in TS handling. Over 530 tons of water are removed during the evaporation of TS, and TS achieves a moisture content of approximately 73%, with dry mass flows approximately unchanged. TS dry mass flow changes from 48524 kg per hour after WS centrifugation to 47770 kg per hour after oil extraction.

Company 3 works with backset ratios within 48 to 52% based on the volume of TS being redirected to anaerobic digestion. The effect of backset on concentrated thin stillage decreases the dry mass flow to 23885 kg per hour, and total and phytate-P flows are 567 and 224 kg per hour, respectively. CDS is combined with WDG to produce WDGS at a mass ratio of 29% and 71%, respectively. Due to the phosphorus-rich composition of CDS, the concentration of P in WDGS increases significantly when compared to WDG (2.7 fold). The

flow of Total-P in WDGS decreases 44% when compared to WS. WDGS is dried to produce DDGS, and its P and dry mass flow remain relatively steady. DDGS is produced at a rate of approximately 95 metric tons per hour, corresponding to 861 kg of Total-P per hour, in which 42% is present as phytate-P.

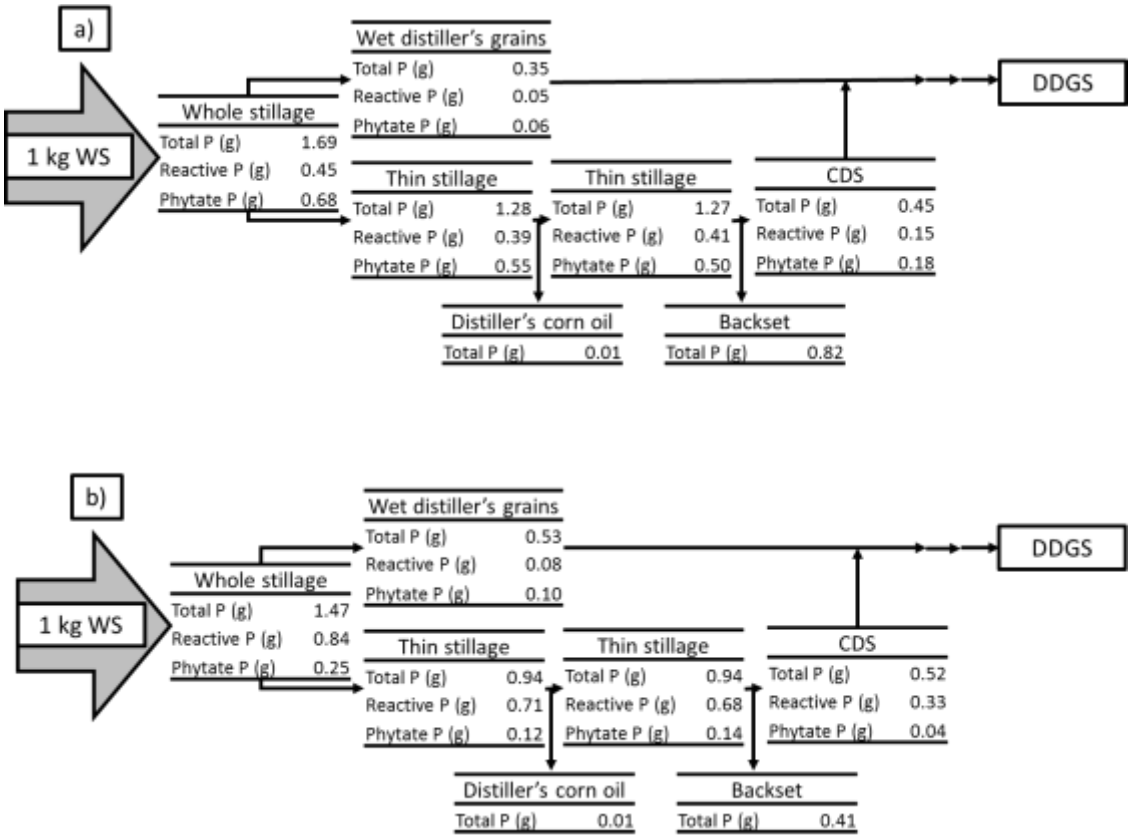
### 3.3.4. Mass balance comparing low- and high-phytate coproducts

A normalized mass balance for phosphorus compounds in dry-grind downstream for companies 1 and 2, i.e., a company with little to no phytase addition (1) and one with addition of phytase during fermentation (2). The data acquired by characterizing the different forms of P in downstream processing when comparing those from a company with no phytase addition to those with addition of phytase are added into a mass balance model. Results are summarized on figure 3.3.

Four key differences have been noticed across the samples from these companies: (i) Phytase addition to fermentation increases Reactive-P concentration in Whole Stillage; (ii) Phytase addition increases the concentration of Total-P and Reactive-P in Wet Distiller's Grains; (iii) Backset of Thin Stillage generated from a system with addition of phytase recycles less total-P; and (iv): DDGS generated from the system with phytase addition has higher concentration of Total-P. One similar fact estimated to these companies (v) is that oil extraction do not significantly decrease Total-P on the downstream process.

(i) Phytase addition to fermentation increases Reactive-P concentration in Whole Stillage: the addition of phytase to the fermentation stream has been tested by some research groups (Khullar et al. 2011; Noureddini and Dang 2009), (Noureddini and Dang 2010)and has been hypothesized to be a positive factor, mostly by decreasing the need of supplementation of external phosphorus sources for the yeast growth and fermentation, as well as by decreasing the chelating property of phytate to essential minerals in the fermentation tank.

Figure 3.3.: P flow within a plant without phytase addition (a), with phytase




addition (b)

Phytase price has significantly decreased in recent years(Wealleans et al. 2016), which has attracted several companies to apply this technology to their dry-grind

systems. As a direct consequence of phytase application, the ratio between reactive-P and total-P in Whole Stillage increases from 26.62% to 57.1% comparing results from a company with little to no exogenous phytase addition to one with phytase addition in the fermentation tank.

(ii) Phytase addition increases the concentration of Total-P and Reactive-P in Wet Distiller's grains: As described in (i), Whole Stillage derived from a system with addition of phytase has significant more reactive-P than the counterpart of a system without phytase addition. Yeast cells are degraded during the initial stages of downstream processing(Han and Liu 2010), dividing its contributions to WDG and TS. It is hypothesized that yeast cells are not degraded to a point in which that most of it becomes soluble during the distillation process. Thus, an assumption is raised regarding a significant ratio of the reactive-P present in Whole Stillage being aggregated to debris of yeast cells. These yeast cells are separated as part of the solid fraction, as WDG, in the centrifugation unit. While phytate-P is not fully utilized directly by yeast cells(Lambrechts et al. 1992), it is present within or aggregated to corn-derived soluble particles(Khullar et al. 2011), which, after the centrifugation is sent to the liquid fraction, i.e., TS. Another possible factor in the centrifugation unit that would likely yield a greater concentration of reactive-P and total-P associated with solid particles, as part of WDG, is the formation of struvite. Struvite formation has been an issue in ethanol downstream processing, especially in thin stillage processing (Johnson and Young 2011). However, there is no reference in describing struvite precipitates

as part of WDG. As struvite is insoluble(Doyle and Parsons 2002), it would likely be separated or aggregated into solid aggregates. These solids, during the centrifugation, would be sent to the solid-rich fraction, i.e., WDG. The mass balance proposed indicate an increase of total-P in WDG by 51.4%, when compared to the solids generated from the system without phytase addition.

(iii) Backset of Thin Stillage generated from a system with addition of phytase recycles less total-P: Due to the flow of Total-P and reactive-P increase significantly to the solid phase after centrifugation, as mentioned in (ii), less P is redirected to the liquid phase, i.e., to Thin Stillage. Backset of Thin Stillage is a common practice in many ethanol plants nowadays, which has proven to have several positive benefits: as energy generation from $CH_4$ production, nutrient and water recycling to the fermentation tanks, and overall, decrease in operational cost over time. Backset, which is an industrial name for anaerobic digestion of Thin Stillage, occurs by transferring some of Thin Stillage to a digester, and decreasing the overall volume of Thin Stillage processed as CDS, and eventually, in DDGS. Our mass balance calculations indicate the backset of a system without phytase addition to recycle approximetaly 100% more P, than in a downstream system with phytase addition (0.82 g P per kg of Whole Stillage and 0.41 g P per kg of Whole Stillage, respectively). Under the assumptions of Total-P being converted to Reactive-P under an anaerobic digestion system, our mass balance results indicate an overall higher rate of P recyclability within an ethanol production facility.

(iv) DDGS generated from the system with phytase addition has higher concentration of Total-P: As a consequence of the hypothesis described in (ii) and (iii) and in the characterization obtained by the mass balance model, less phosphorus is recycled to the fermentation tank in a system with backset when phytase is added. As a direct effect of solids being richer in Total-P in a system with phytase addition, and knowing that these solids, as Wet Distiller's grains, are not redirected to P recycling in the backset process, more Total-P is redirected to the formation of wet distiller's grains with solubles. Wet distiller's grains with solubles are dried to produce DDGS, which carry more Total-P per mass of Whole Stillage processed, when a system with phytase is considered (1.02 g P/kg of Whole Stillage processed) and compared to a system without phytase addition (0.62 g P per kg of Whole Stillage processed).

However, as a direct consequence of phytase treatment, most of the phosphorus present in DDGS is present as reactive-P (69.3% of Total-P as reactive-P), while in a system without phytase addition, reactive-P represents 32.3% of Total-P. A system without phytase addition yields around 0.26 g of Phytate-P in DDGS per kg of Whole Stillage processed (41.9% of Total-P in DDGS), while in a system with phytase addition, phytate-P is present at 0.09 g per kg of Whole Stillage processed (8.8% of Total-P in DDGS).

(v) Distiller's corn oil extraction do not impact significantly P removal from DDGS nor P flow in systems with and without exogenous phytase addition: based on the assumption of distiller's corn oil composition not varying significantly among

different plants, it can be concluded that P removal from Distiller's corn oil is not significant. Considering a case scenario with phytase addition and one without, using an overestimation of 100% oil removal from Thin Stillage, around 0.01 g of Total-P is removed from kg of Whole Stillage processed.

### 3.4. Conclusions

The current changes in the dry-grind ethanol industry directly impact the feed composition of DDGS. A summary using samples from five dry-grind ethanol plants is presented at this article, as well as a phosphorus compositional analysis for each sample. Our samples indicate that some companies apply exogenous phytase in their coproduct processing, most likely to increase nutritional value of DDGS. Addition of exogenous phytase has been a common practice in order to increase the bioavailability of phosphorus in DDGS, as well as a feasible and cheap option to increase ethanol fermentation yields. However, this technique has been known to increase considerably fouling rates in thin stillage evaporation. This article contrasted and compared phosphorus characterization among different plants, and our findings indicate that the addition of phytase not only may increase fouling tendencies in thin stillage processing, but also may recycle less phosphorus to the fermentation process via backset of thin stillage.

# CHAPTER 4: PHYTATE EXTRACTION FROM COPRODUCTS OF THE DRY-GRIND CORN ETHANOL PROCESS

## Outline

Distiller's dried grains with solubles (DDGS), the major coproduct of dry-grind ethanol production, are being increasingly used in the global market as animal feeds for both energy and protein supplement. DDGS contains high levels of phosphorus in the form of phytate, which cannot be digested by monogastric animals, such as poultry and swines, which in turn produce manure with high levels of phosphorus. Phytate is a highly-valued chemical and can be recovered from DDGS precursors in the downstream processing of dry-grind coproducts. This study was aimed at the utilization of the AG 1-X8 anion exchange resin to remove and purify phytates from thin stillage, and near 100% efficiency of adsorption and over 90% desorption from the resin beads was achieved. The end product showed a similar profile to the standard phytate, and could be precipitated and crystallized as calcium phytate. This process may potentially bring benefits to the parties involved, more revenue to corn ethanol facilities, improved digestibility of animal feeds, and minimized environmental impact, as less manure with a high content of phosphorus is applied to the soil.

## 4.1. Introduction

With the recent expansion of the U.S. corn ethanol industry and fluctuations in the corn and ethanol markets, the coproducts generated from ethanol separation processes are playing a vital role in the overall economic turnout of corn ethanol facilities and are saturating the global markets with feeds for energy and protein

replacement (Wang et al. 2015). In a typical dry-grind processing, corn is directly milled without soaking in water and then enzymes and yeast are sequentially added for the ethanol fermentation (Luangthongkam et al. 2015). This process is characterized for its high versatility and low capital investment. The fermentation broth is then processed by distillation, such that ethanol is separated and the residue is the whole stillage (WS). WS is composed of proteins, fibers, residual sugars, lipids, and other minor components, derived from both the yeast cells and unfermented corn residues (Liu and Han 2011). WS is centrifuged and separated into two streams: thin stillage (TS), a liquid phase with over 90% of moisture content, and wet distillers grains (WDG), which is the heavy fraction, with a solid content of around 50%. Throughout the several possible procedures found in industrial plants, TS is usually evaporated and concentrated to achieve a lower moisture content, producing industrial “syrup”, often labelled as condensed distillers with solubles (CDS). This syrup is then mixed with the solids present from WDG, and then dried at high temperatures to generate distiller's dried grains with solubles (DDGS) (Alkan-Ozkaynak and Karthikeyan, 2012). In order to increase the shelf life of DDGS for commercialization, the drying process of DDGS is crucial. During the pre-processing of the corn grains the dry-grind process is conducted by physical methods such as hammering, and thus the generated coproducts usually contain detectable levels of starch and impurities (Zabed et al. 2017). There are often additives in this process, such as enzymes and vitamins, which can increase the overall nutritional value of DDGS (Rausch

and Belyea, 2006).

There has been a growth in the production and commercialization of DDGS due to the rapid expansion of the dry-grind process throughout the ethanol industry. Due to the nutritional value present in DDGS, it quickly gained attention from the markets and in 2015 achieved a significant share, about 23%, of the non-ruminant animal feeding markets, such as poultry and swine industries (RFA, 2016). DDGSs are also an important source of minerals and are known as a feeding material rich in potassium, magnesium, zinc, sulfur, and phosphorus (P) (Batal and Dale, 2003; Belyea et al. 2006). As regards to P, this nutrient is often regarded as one of the most economic value components in animal feeds. However, Spiehs et al. (2002) reported too high levels of P in DDGS, with concentrations as high as 10 g of P per kg on dry basis. Such concentration is higher than the requirements of most ruminant animals (Rausch and Belyea, 2006). In DDGS, the largest portion of organic P is found as phytate. This molecule cannot be directly assimilated by non-ruminant animals due to the lack of microbial phytases in their digestive system (Cromwell et al. 1995). Feeding non-ruminants with phytate-rich materials, such as DDGS, will further increase the P content in manures, which are often applied as fertilizers by farmers (Spiehs et al. 2010). These high levels of P in soil typically exceed the soil binding capacity for phosphates and other forms of P ions, potentially resulting in high levels of P in runoff water. The major environmental impact of such an effect is the eutrophication of surface and underground water bodies (Belyea et al.

2006; Koelsch and Lesoing, 1999; Noureddini et al. 2009).

In corn, P is mostly present as inositol-phosphates (Reddy et al. 1982). One molecule of inositol can bind up to six phosphate anions. The fully substituted molecule, inositol-hexaphosphate (IP6), is often referred as phytic acid, or known by its salt name, phytate. According to Ravindran et al. (1995), phytate is present in levels ranging from 50% to 80% of the total P present in corn. Phillippy et al. (1987) evaluated the potential of industrial yeasts for hydrolyzing phytate during ethanol fermentation, and reported that these cells are able to produce phytase. This enzyme is able to degrade phytate to the forms of inositol mono-, bis-, tris-, tetra-, and pentakisphosphates (IP1, IP2, IP3, IP4, and IP5), as well as inorganic phosphate. The global market for phytase has grown significantly over the past years, resolving specific demands for monogastric animals, and its market value reached over 700 million US dollars in 2015. (Bhavsar and Khire, 2014) However, according to Liu and Han (2011), in corn-to-ethanol fermentation systems without exogenous phytase addition, about 40% to 50% of P in the fermentation mash usually remains as phytate.

Several attempts to increase the degradation of phytate throughout or after the fermentation have been recently reported in the literature. Noureddini and Dang (2009) added phytase from *Aspergillus niger* to catalyze phytate hydrolysis in WS, whereas Khullar et al. (2011) proposed a step of incubation with phytase prior to corn saccharification. Despite the environmental problems posed with phytates in the corn ethanol industry, this molecule is often seen as a high-value

chemical in the market. Highly employed by some sectors of the food market and textile industries, phytate exhibits strong chelating and preservative properties (Angel et al. 2010; Cupisti and Kalantar-Zadeh, 2013) and an excellent inoxidizability (Minihane and Rimbach, 2002) being often used as food additive, preservative, and antioxidant. In recent years, the beneficial effects of phytates have been highlighted and explored by the pharmaceutical industry, particularly in the prevention of renal calculi (Saw et al. 2007), diabetes (Lee et al. 2006), some types of cancer (Vucenik and Shamsuddin, 2006), and Parkinson's disease (Xu et al. 2008)

If phytate could be extracted from DDGS, it will be a win–win situation for both the industrial sector and environmental protection. The industrial process for phytate manufacturing consists of acid extraction from rice bran or cereals, followed by a chemical precipitation step. Strong acids are needed to dissolve phytates in a traditional phytate extraction (Canan et al. 2011). If phytate was to be recovered from end products, such as DDGS, the low pH of the extracted product would likely make it inappropriate as a feeding material. Ion exchange techniques have been applied to extract P products from liquids (Lehrfeld, 1989; March et al. 2001; Park et al. 2006). However, little has been done on phytate extraction from corn ethanol coproducts. This study assayed a particular type of anionic resin, AG 1-X8, and the critical operational conditions to maximize phytate extraction from dry-grind coproducts.

## 4.2. Materials and methods

### 4.2.1. Sample collection, storage and chemicals

WS, TS, WDG, CDS, and DDGS were obtained from a large-scale dry-grind corn ethanol plant located in the state of Iowa, USA. All samples were stored in the refrigerator at −20 °C prior to use. The AG 1-X8 anion exchange resin (100–200 mesh, chloride form) was purchased from Bio-Rad Co. (Berkeley, CA). All the other chemicals were of analytical grade.

### 4.2.2. Sample analysis and preparation

All samples were analyzed for moisture content (wet basis), total P, and phytate P. Moisture content was defined as the ratio between the weight loss after drying at 105 °C and the wet weight. For total P determination, samples were dry-ashed, and the colorimetric assay for P was performed in the digests. The samples were analyzed with an adapted molybdate-blue method, based on P test kits (Hach, Loveland, CO). An acid extraction with 1 mol L−1 HCl was performed prior to phytate analysis. In order to precipitate the P salts, the pH of the liquid phases, containing soluble phytates, was changed to neutral by treatment with 1 mol $L^{-1}$ $CaCl_2$ solution. P salt sediments were then washed, dried and redissolved in 1 mol $L^{-1}$ HCl, for which analysis on total P and phosphate P by the molybdate-blue method were conducted. Phytate P content was defined as the difference between total P and phosphate P. All chemical analyses were conducted in triplicate.

### 4.2.3. Batch extraction: adsorption and desorption

The AG 1-X8 resin was equilibrated with 2 mol $L^{-1}$ HCl, and then washed with distilled water, promoting the pH change to neutral. Then, 10 mL of CDS or TS solution were added to a 25 mL glass vial containing 3 g of the resin. Adsorption

efficiency was defined as the ratio between the P concentration in the supernatant and the initial P concentration in the liquid phase. Throughout full factorial experimental design, effect of time, temperature, and pH on adsorption were investigated.

For evaluating desorption, the resin beads were removed by filtration and added to various eluent solutions at different concentrations. These eluents (HCl, NaOH, and NaCl) were intended to elute the retained P anions from the resin beads. Desorption efficiency was calculated as the ratio between P concentration in the supernatant and total P amount adsorbed on the resin beads.

### 4.2.4. Column extraction – adsorption and desorption

A chromatographic glass column (1.0 cm × 30 cm) was used as support for the AG 1-X8 resin. The resin beads, loaded at different weights, were equilibrated with HCl 2 mol $L^{-1}$ and washed with distilled water until the pH in the eluted water was neutral. The liquid phase of TS was used as the target solution, which was added sequentially with a loading increment of 10 mL, i.e., each 10 mL of solution added and adsorbed was analyzed in terms of reduction of the P level. Prior to desorption, the resin beads were washed with distilled water for removal of impurities.

For the desorption experiments, the desorption agent solution was loaded sequentially in volumes of 10 mL, i.e., each 10 mL of solution added was analyzed in terms of concentration of P compound. Desorption efficiency was defined as the increment in P concentration over the amount of P adsorbed on the resin beads.

#### 4.2.5. Calcium phytate precipitation and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) analysis

Phytate precipitation was carried out by adding excess $Ca^{2+}$, as $CaCl_2$, followed by a pH shift to neutral by the addition of NaOH. The obtained sediments were washed with distilled water and dried at 105 °C until constant weight was obtained.

For the ATR-FTIR analysis, spectra were recorded in the 4000 to 400 $cm^{-1}$ range on a Thermo Scientific Nicolet iS50 FTIR spectrometer (Waltham, MA) with a built-in diamond ATR. The background used was ambient air. Each test was scanned 32 times with a resolution of 0.24 $cm^{-1}$. All spectra were normalized. Sodium phytate hydrate from rice (S06880/Pfaltz & Bauer, Waterbury, CT) was used as standard. Sodium phytate samples were obtained using NaCl as the eluting agent, followed by evaporating this solution at 40 °C.

### 4.3. Results and discussion

#### 4.3.1. Moisture content and P analysis

The WS, containing undissolved corn fragments and residual yeast cells, presented a moisture content of 87.94%. During solid–liquid separation of WS, most solids in WS are redirected to WDG. TS had a moisture content of 95.71%, whereas WDG had 49.75%. In CDS, concentrated from TS through series of evaporations, a moisture content of 70.48% was achieved. CDS has a viscous texture, and moisture contents lower than 70% are economically unfeasible,9 mainly due to the increase in suspended solids and osmotic pressure. The dry blend of CDS and WDG was dried to produce DDGS. DDGS samples used in this study presented moisture contents of about 14.92%, within the expected

range reported to prevent DDGS from becoming moldy and unusable (Bhadra et al. 2007).

WS had a total P concentration of 12.02 mg $g^{-1}$, of which 40% was found to be phytate P, with a concentration of 4.81 mg $g^{-1}$. After the first solid–liquid separation, the concentrations of total P and phytate P (23.57 mg $g^{-1}$ and 10.11 mg $g^{-1}$, respectively) in the TS had doubled, whereas WDG presented much lower concentrations of total P and phytate P (10.21 mg $g^{-1}$ and 3.27 mg $g^{-1}$, respectively). The difference was most likely attributed to the fact that more P went to the liquid phase (TS) than the solid phase (WDG) during the solid–liquid separation of WS, which primarily indicates that most P in the streams is soluble (Liu and Han, 2011). The water removal step on TS to produce CDS maintained similar levels of total P (23.77 mg $g^{-1}$) and showed a slight decrease of phytate P (9.41 mg $g^{-1}$). Due to the recycling of a TS portion as a backset for the cooking step, the concentrations of both total P and phytate P in DDGS (10.59 mg $g^{-1}$ and 4.50 mg $g^{-1}$, respectively) were just a little lower than those of WS. Comparing with the literature results,4 similar values were found, particularly for fractions of TS (19.4 mg $g^{-1}$), CDS (18.8 mg $g^{-1}$), and WS (11.0 mg $g^{-1}$) in terms of total P. As shown in Table 1, TS was the stream with the highest moisture content in the downstream processing of dry-grind coproducts.

Table 4.1.: Moisture content, total P, and phytate P in dry-grind coproducts

| Sample | Moisture content (%) | Total P (mg $g^{-1}$) | Phytate P (mg $g^{-1}$) |
|---|---|---|---|
| WS | 87.94 ± 0.15 | 12.02 ± 0.60 | 4.81 ± 0.01 |
| TS | 95.71 ± 0.13 | 23.57 ± 1.12 | 10.11 ± 0.66 |

| | | | |
|---|---|---|---|
| WDG | 49.75 ± 0.40 | 10.21 ± 0.53 | 3.27 ± 0.21 |
| CDS | 70.48 ± 0.22 | 23.77 ± 0.23 | 9.41 ± 0.34 |
| DDGS | 14.33 ± 0.39 | 10.59 ± 0.20 | 4.50 ± 0.15 |

Corn grain presents high phytate levels, with as much as 90% of organic P found as phytate (Boyer et al. 1987) representing overall levels of around 75% of the total P found in the kernel (Abelson, 1999). Assuming that the P present in corn is not a limiting factor in the ethanol fermentation, and thus, no extra P is added to the fermentation media, it is observed that phytate levels decrease when comparing ethanol coproducts and corn. Andlid et al. (2004) attributed this to the expression of a type of phytase enzyme by *Saccharomyces cerevisiae* during the fermentation. We assumed that the combination of acidic pH, high temperature during processing, and the presence of endogenous phytase in the fermentation tank favor the hydrolysis of the phytate present in corn. Even though phytate levels are decreased on a relative basis, Liu and Han (2011) described several-fold increase in nutrients during the downstream processing of dry-grind ethanol plants. Thus, phytate, even though is degraded during fermentation, is still found at relative large concentrations due to nutrient increase, as described by Liu and Han (2011).

### 4.3.2. Evaluation of phytate extractants

Experiments of phytate extraction with acid or water (Fig. 1) show that phytate concentrations in acid and water extracts from CDS differed by only 6.2% (1026 mg $L^{-1}$ for the acid extract, and 966 mg $L^{-1}$ for the water extract), with a significance level of 0.06. Similar results were reported, with 82% of the total P in

WS dissolved in the liquid phase (Noureddini et al. 2009). Since the majority of phytates in the streams were present as the soluble form, the extraction with strong acids, which brings additional cost and damage to the raw material and barely provides any significant process gain, could be avoided. Thus, the feeding value of the coproducts could be maintained by introducing a mild extraction method.

Latta and Eskin (1980) and Haug and Lantzsch (1983) have developed extraction methods for phytate in cereals. Their methods include an acid pretreatment of phytate-rich grains, using dilute HCl, which has proven to dissolve the phytate present in granules into the bulk solution phase. The results of this study showed that most phytate may have already been dissolved in the liquid fraction due to the low pH (4.42) of CDS. In addition, the pH of TS and WS were 4.47 and 4.48, respectively, which meant phytate in TS and WS were likely to be present in its soluble form, as evidenced from the significant increase in concentration of this substance in the liquid phase (TS).

Batch extraction of phytate from CDS at different dilutions (Table 2) showed significant variability among samples with different moisture contents (p-value <0.05 for total P and phosphate). Controlling the moisture content of CDS by water addition reflected the concentration of P in the samples. Resin AG 1-X8 showed a better adsorption performance on P solutions with lower concentrations, achieving a 64.51% adsorption of the total P at a moisture content of 95%, whereas only 43.92% of the total P was adsorbed at a moisture

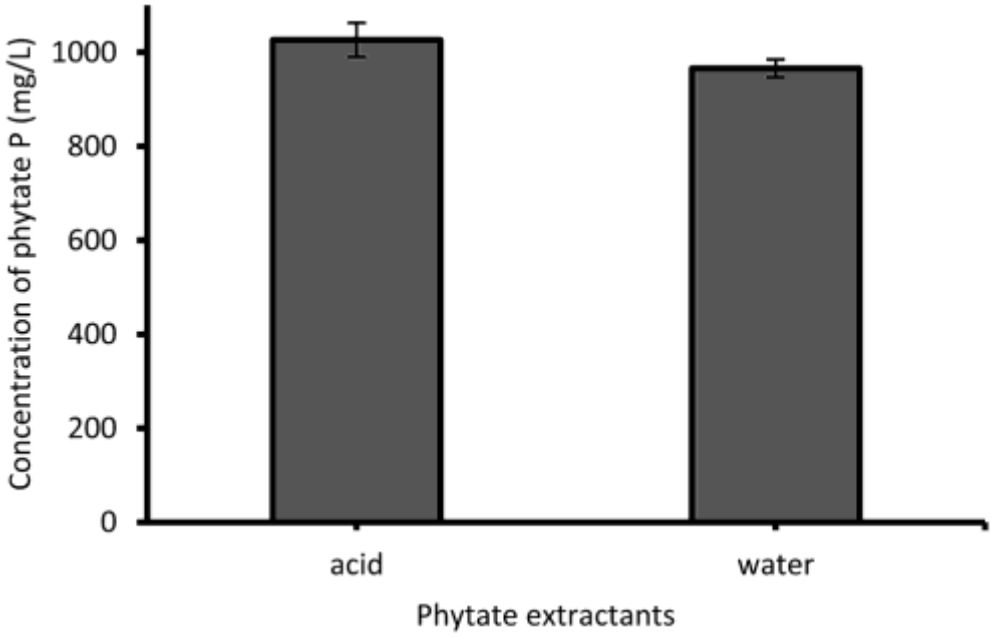
content of 75%. Solid–liquid adsorption occurs in four steps: liquid phase mass transfer, interface diffusion between the liquid phase and the exterior surface of the adsorbent, intrapellet mass transfer involving pore diffusion and surface diffusion, and an adsorption–desorption reaction. The increase in viscosity and osmotic pressure effects followed by a decrease in moisture content is hypothesized to be a key factor in the liquid phase mass transfer and on the pore diffusion and surface diffusion processes involved in ion exchange.

Table 4.2.: Effect of moisture content, temperature, time, and pH on P adsorption

| | | **Adsorption efficiency (%)** | |
|---|---|---|---|
| **Factor** | **Level** | **Phosphate** | **Total P** |
| Moisture content of CDS (%) | 95 | 50.7 | 64.51 |
| | 90 | 46.42 | 59.13 |
| | 85 | 37.7 | 54.47 |
| | 80 | 33.83 | 49.85 |
| | 75 | 31.43 | 43.92 |
| Temperature (°C) | 4 | 34.83 | 62.65 |
| | 30 | 38.86 | 64.26 |
| | 40 | 39.53 | 63.02 |
| | 50 | 39.32 | 63.68 |
| | 60 | 38.68 | 63.24 |
| Time (min) | 10 | 38.68 | 64.26 |
| | 20 | 39.53 | 64.49 |

| | | | |
|---|---|---|---|
| | 30 | 38.89 | 64.39 |
| | 60 | 40.17 | 64.19 |
| | 120 | 38.98 | 64.42 |
| | 1440 | 39.53 | 63.97 |
| pH | 1 | 22.85 | 47.01 |
| | 2 | 28.39 | 57.29 |
| | 3 | 31.73 | 59.26 |
| | 4 | 33.54 | 63.09 |
| | 5 | 35.27 | 64.19 |
| | 6 | 39.92 | 64.17 |

Figure 4.1. Effect of moisture content on the batch extraction of phytate by anion




exchange

The highest adsorption efficiency of phosphate and total P was attained on

diluted CDS with 95% moisture content, which was very close to that of TS (95.71%). In this process, approximately 75% of the total P flows into TS after centrifugation of WS (Alkan-Ozkaynak and Karthikeyan, 2012). If most phytate in TS could be extracted, the final content of phytate in DDGS would be drastically decreased. Furthermore, due to the composition of TS, most residual solids, which are dead yeast cells and small corn residue particles (Liu et al. 2007) can be separated by a simple filtration, centrifugation or decantation. Therefore, subsequent extraction experiments were focused on TS.

### 4.3.3. Effect of temperature and time on the batch extraction of phytate by anion exchange

The adsorption efficiency for TS, an extract with high concentrations of total P, presented a constant value of 63.4% for total P ($p$-value < 0.01) and a desirable lower efficiency of 38.1% for phosphate ($p$-value < 0.05), across the range of temperatures studied, as shown in Table 2. The robustness of this resin, under the conditions studied, is highly desirable for P extraction because, according to Lee et al. (2011), processing of TS in a dry-grind ethanol plant occurs within a narrow range of temperatures close to 70 °C.

The results for adsorption potential were constant at the time range studied (Table 2), from 10 min to 1440 min, achieving a constant value of 64% ($p$-value < 0.05). These results may suggest that the adsorption equilibrium occurred within 10 min at the conditions studied. Previous chromatographic separations of phytate using similar types of resins and conditions achieved good separation of phytate within 30 minutes (Latta and Eskin, 1980) or even with a separation

column without control of residence time.36 The high moisture content and the amount of resin in contact with the bulk suspension of stillage allow a high adsorption rate, as predicted by Latta and Eskin (1980).

### 4.3.4. Effect of pH on the batch extraction of phytate by anion exchange

The results at the bottom of Table 2 showed better adsorption for both total P and phosphate at higher pH values within the acidic region. At extremely acidic conditions, such as pH 1, 2, and 3, total P was adsorbed at 47.01%, 57.29%, and 59.26%, respectively. Similar to phosphate, the values were 22.85%, 28.39%, and 31.73%. For pH values ranging from 4 to 6, a plateau region was obtained for the adsorption of total P, attaining a circa 63% adsorption on the resin beads. For phosphate, however, there was still an increase in the adsorption within this range with the increment of pH, with values of 33.54%, 35.27%, and 39.92% at pH values of 4, 5, and 6, respectively. The highest P adsorption levels were achieved at higher pH values. Kaufman and Kleinberg (1970) claimed that one of the most important factors for adsorption in anionic resins was the solution anion electronegativity. Phytate has six protons with pKa values of 2.18, two with pKa of 5.73, and two with pKa equal to 9.21 (Evans et al. 1980). Therefore, a high pH will favor the ionization of phytate by dissociating $H^+$ from the hydroxyls, resulting in an increase of electronegativity in phytate, which in turn will increase the ability of phytate to participate in the anion exchange (Kaufman and Kleiberg, 1970). However, the precipitation that occurs in TS when pH is over 7 would hinder the anion exchange. The resin presented a good adsorption performance when the pH was from 4 to 6 (Table 2). Since the pH of the TS (4.47) was within this

range, no pH adjustment was needed before adsorption.

### 4.3.5. Phytate desorption from the anion exchange resin

NaCl exhibited a better desorption performance on adsorbed phytate at both 0.5 and 1.0 mol $L^{-1}$ concentrations as compared to HCl and NaOH (Table 3). Table 4 shows that the highest desorption efficiency for phytate was obtained when the concentration of NaCl was over 1.5 mol $L^{-1}$. Thereby, 1.5 mol $L^{-1}$ NaCl was used as eluent in subsequent column phytate extraction experiments. This can be explained by the displacement of adsorbed phosphorus anions by chloride anions from the eluate solution. The better performance of NaCl as a desorption agent agrees with a previous methodology for phytate extraction from cereal grains, in which Latta and Eskin (1980) used NaCl to selectively desorb phytate over inorganic phosphate from a similar anion exchange system. Helfferich (1962) described that NaCl is preferably used as a desorption agent, or even regeneration agent in some conditions, due to its low-toxic potential and low cost.

Table 4.3.: Effect of various eluents with different concentrations on the desorption efficiency of P

| Desorption agent | Conc. (mol $L^{-1}$) | Desorption efficiency (%) | | |
|---|---|---|---|---|
| | | Phosphate | Phytate | Total P |
| HCl | 0.5 | 65.96 | 26.15 | 26.15 |
| | 1 | 69.96 | 45.39 | 46.24 |
| NaCl | 0.5 | 60.47 | 33.87 | 36.24 |
| | 1 | 75 | 83.69 | 77.33 |
| NaOH | 0.5 | 47.73 | 9.66 | 16.35 |
| | 1 | 69.4 | 35.55 | 37.34 |

Table 4.4.: Effect of different NaCl concentrations on the desorption efficiency of P

| NaCl concentration (mol $L^{-1}$) | Desorption efficiency (%) | | |
|---|---|---|---|
| | Phosphate | Phytate | Total P |
| 0.05 | 36.15 | 2.68 | 9.44 |
| 0.1 | 48.97 | 3.09 | 12.43 |
| 0.25 | 57.81 | 8.21 | 18.17 |
| 0.5 | 60.47 | 33.87 | 36.24 |
| 1.0 | 72.39 | 81.96 | 73.97 |
| 1.5 | 83.98 | 98.52 | 88.26 |
| 2.0 | 80.6 | 97.5 | 86.87 |

**4.3.6. Column extraction**

The column extraction results shown in Fig. 2, demonstrate a higher adsorption rate for phytate P than that for phosphate P. One of the most plausible theories could be the higher concentration of negatively charged oxygen atoms in phytate than in phosphate. The adsorption efficiency of phytate was sometimes over 100%, and this excessive contribution was probably due to the presence of P in other forms being considered as phytate and also adsorbed on the resin.

The scope of the adsorption curve shows a gradual decrease in the adsorption efficiency, explained by saturation of the resin beads with adsorbed ions. The results confirmed this theory since the higher the resin load, the higher the absolute saturation potential was determined to be. This increase might have

resulted from the longer adsorption time caused by a higher amount of resin in the column.

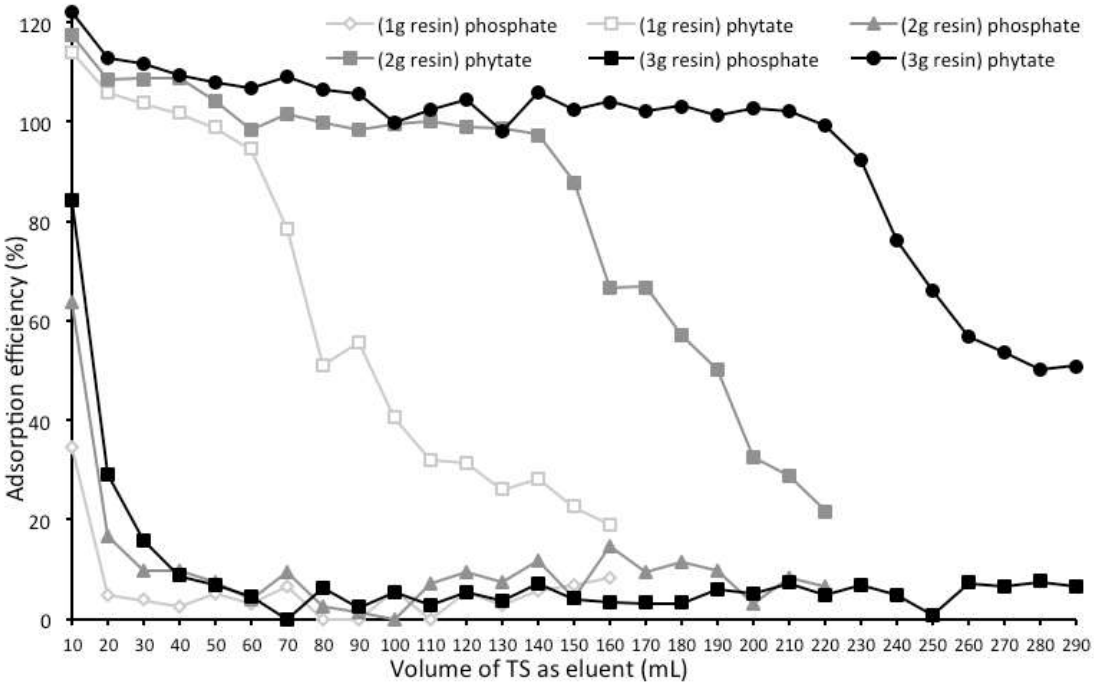



Figure 4.2.: Adsorption of phosphate and phytate during column extraction

For the column containing 3 g of AG 1-X8 resin, approximately 100% of total phytate and 6.15% of total phosphate in TS were adsorbed, using 220 mL of TS as elution agent. The results also showed a recovery of about 91% of adsorbed phytate using 1.5 mol $L^{-1}$ NaCl as the desorption agent, which had a total volume of 30 mL (Fig. 3). As a direct consequence of this, the eluate solution showed a significant increase in the concentration of phytate, when compared to its original concentration in the dry-grind coproducts. This increase in the concentration could be conducive to harvest phytate via chemical precipitation.

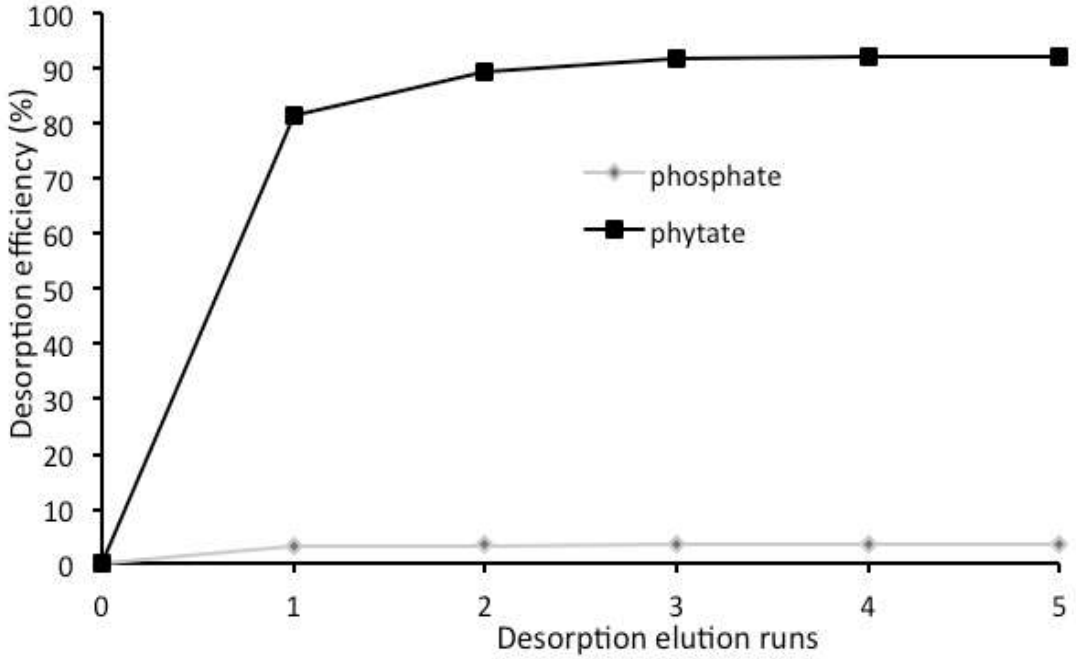



Figure 4.3.: Desorption of phosphate and phytate during column extraction.

### 4.3.7. ATR-FTIR analysis of extracted phytate

The ATR-FTIR spectra of standard sodium phytate and extracted sodium phytate are shown in Fig. 4. A weak absorption band around 1645 $cm^{-1}$ and a broad absorption band at 3400 $cm^{-1}$ (data not shown) were attributed to O–H bonds of the adsorbed water molecules (Celi et al. 1999). The absorption band around 1398 $cm^{-1}$ was assigned to the C–O stretching vibration (Ganesan and Epple, 2008). Two bands around 1186 and 496 $cm^{-1}$ are attributed to the PO43− groups. The bands around 1038, 986, 909, 850 and 793 $cm^{-1}$ were ascribed to C–O–P vibrations from various types of phytate (IP2–IP6).44 The small shifts in the vibrational bands between the two types of sodium phytate were possibly due to the residual sodium chloride mixed in the extracted one and different types of

phytate in the samples (Ganesan and Epple, 2008).

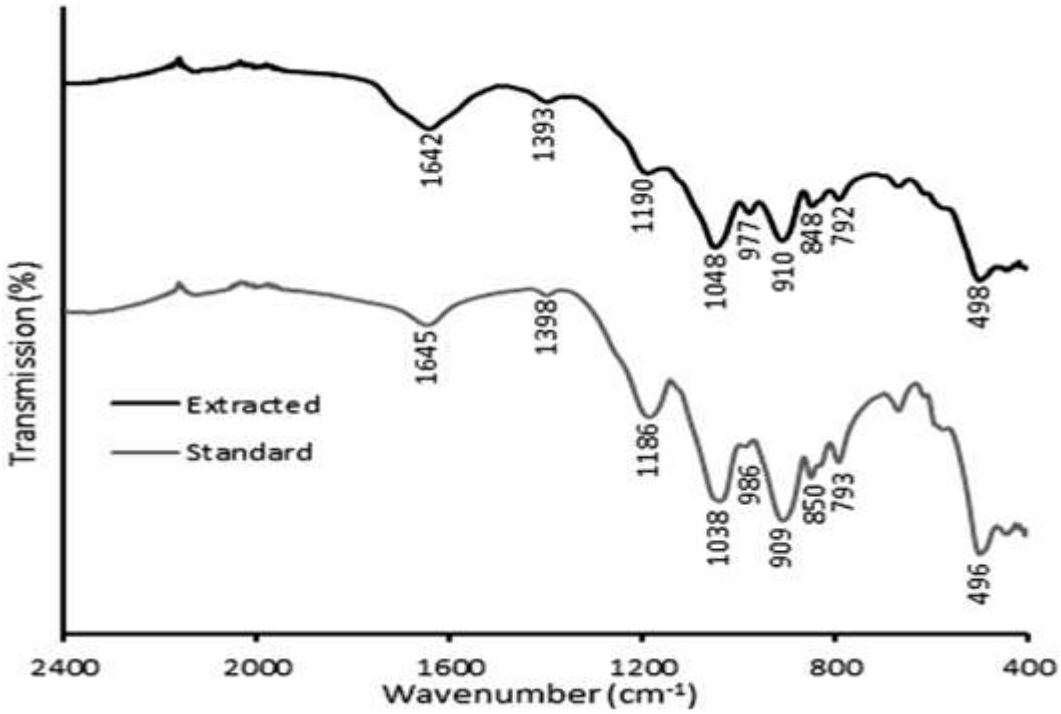



Figure 4.4.: FTIR spectra of sodium phytate from the extractions and standard sodium phytate from rice bran.

### 4.3.8. Overall yield and significance of the process

Precipitation of phytate with $Ca^{2+}$ ions does not only remove the chloride ions that are simultaneously desorbed, but also promotes the discoloration by the removal of some organic chromophoric molecules, which phytate could have adsorbed after the elution. From the results, 0.49 g of calcium phytate was yielded as a result of the first 30 mL elution. Based on the results, it was calculated that 0.39 g of P as pure phytate, or the equivalent of 1.85 g of calcium phytate, could be obtained from 1 kg of TS with the optimized conditions of this

study.

The approach in this study provided a new method to remove phytate from the coproducts of the dry-grind corn ethanol process. It is expected that this extraction process will not have any significant negative effects on the nutritional value of DDGS as a whole since major nutrients cannot be adsorbed to the column under the conditions studied. For the dry-grind ethanol downstream processing, approximately 75% of the P present in WS is sent to TS. Thus, if most phytate in TS could be extracted, final content of P in DDGS would be drastically reduced. Removal of P from ethanol coproducts has been described before by Alkan-Ozkaynak et al. (2010), where they used aluminium- and calcium-based coagulants to recover P from TS through a chemical-based separation process, resulting in a sludge that could be applied to agriculture fields. This can provide a feasible solution to the P issue in DDGS, but little value is gained after the sludge is applied to the land. On the other hand, extraction of phytate and generation of calcium phytate can potentially yield additional revenue to the ethanol industry, as well as provide a solution to attenuate environmental concerns regarding the eutrophication of water bodies due to P runoff. Usually, P in soil after manure application moves to groundwater bodies and deep soil layers, potentially damaging the aquatic environment by eutrophication (Zhang et al. 2016). This process may provide a great opportunity for the U.S. ethanol industry because extracting phytate from corn ethanol coproducts can create additional revenue while increasing the feeding value of

coproducts and decreasing the P content in animal manure.

### 4.4. Conclusion

With this study, it was proved that it is better to start the extraction of phytate directly from TS since its acid treatment did not result in a significantly larger gain of phytate extraction. The AG 1-X8 resin was proved to be effective within the typical temperature range for TS processing, and the extraction could be accomplished within several minutes. Sodium chloride was proven to be the best desorption agent. Both the AG 1-X8 resin and NaCl have superior specificity for phytate adsorption and desorption over inorganic phosphate. The overall process could yield up to 1.85 g of calcium phytate per kg of TS.

# CHAPTER 5. RETROFIT PROCESS DEVELOPMENT FOR PHYTATE EXTRACTION FROM CORN-ETHANOL

# COPRODUCTS USING INDUSTRIAL ANION EXCHANGE RESINS

## Outline

Phytate, the principal storage form of phosphorus in corn, is found to remain as a significant portion of phosphorus in the corn-ethanol coproducts streams. This poses concerns over poor digestion of these coproducts by monogastric animals and subsequent environmental issues on phosphorus. An ion-exchange based process was developed to extract the phytate from thin stillage, which was selected as the influent based on the inositol phosphate distribution conducted on different corn-ethanol coproducts. Commercial industrial resins (IRA-93, IRA-68, IRA-900, IRA-400 and IRA-402) were characterized and screened for the phytate adsorption, specificity and capacity. Different types of eluents (NaOH, NaCl, $NaHCO_3$, HCl, and $NH_4OH$) were tested to evaluate the desorption capacity in all the six resins. Phytate-P isotherms and kinetics of the adsorption process, breakthrough profile on IRA-900 column and phytate elution curves were presented. This process yields a phytate-rich solution (11 g $L^{-1}$), which is 25 fold concentrated compared to the concentration in thin stillage. The results show that the proposed retrofit process allows to leverage the existing corn-ethanol plants for high-value phytate production.

## 5.1. Introduction

Commercial production of fuel ethanol involves breaking down the starch present in corn to simple sugars, followed by the bioconversion of sugar molecules to ethanol via yeast fermentation. The downstream processing involves the recovery of ethanol by distillation and the residual coproducts are further processed to serve as animal feed. Accounting for over 80% of the total ethanol production in the United States, dry-grind ethanol processing differs from the wet-milling process by the absence of an initial steeping operation to the corn grain(Rausch and Belyea 2006). Most of the coproducts of interest are separated before the fermentation process in wet-milling industries. In the dry-grind process, the residual components are separated after the fermentation and distillation.

In a typical dry-grind plant, coproduct recovery starts with whole stillage (WS), which accounts for the bottom fraction of ethanol distillation from fermented mash. WS, containing 6-16% of total solids, is a hot, acidic, and viscous fluid mixture, with limited shelf life. WS is usually dried for easier handling, storage, and end use. The most common practice to handle WS and transform it into a stable product consists on a series of separations. The first step is a solid-liquid separation, where the solid fraction from this separation is known as wet distiller's grains (WDG), and the liquid fraction, which contains about 90% to 95% of moisture, is thin stillage (TS). TS is processed into multiple-effect evaporators to produce Condensed Distillers Solubles (CDS), reducing its moisture content to about 60 to 75%. CDS is then combined with WDG to produce a nutrient-rich material, wet distiller's grains with solubles (WDGS), which is dried in order to produce dried distiller's grains

with solubles (DDGS), the common commercial coproducts on the global feed market(Rausch and Belyea 2006).

DDGS has high concentration of phosphorus (P), and often times P may be overdosed in situation in which DDGS is used above the recommendation limits for each animal(Spiehs et al. 2002). This may indirectly yield an increase in P excretion by animals(Noureddini et al. 2009). Alkan-Ozkaynak et al. (2010) developed the first study reporting in utilizing chemical coagulation and flocculation as a treatment for TS, in order to reduce P concentration. According to Alkan-Ozkaynak et al. (2010), the majority of P and solids in TS are in the dissolved form (<0.45 µm), which, if centrifuged, would yield a solid fraction with high crude protein and low P concentration.

Phytic acid (known as inositol hexakisphosphate (IP6), inositol polyphosphate, or phytate when in salt form), is the main storage form of phosphorus in many plant tissues in many seeds, grains, and cereals(Maga 1982). According to Ravindran et al. (1994), phytate is present in levels ranging from 50% to 80% of the total P present in corn. Phytate has a wide range of applications in many fields, especially due to its strong chelating property. Examples are the (1) Utilization as antioxidant agent in the food industry, since it is able to suppress oxidative reactions catalyzed by iron, which improves food shelf life, and can help reduce the risk of some inflammatory bowel diseases(Graf et al. 1987); (2) Utilization of lower inositol phosphates by medicine-related industries, since it is known that these can have a function in second messenger transduction systems (Fox and Eberl 2002), as

well as the use in decreasing effects of gastritis, duodenum inflammation, and diarrhea (Hambidge 1992); (3) use as anti-corrosion agent due to its strong metal-complex potential energy, which provides a non-poisonous and easily-controlled galvanization process (Neevel 1999); and (4) the use by plastic manufacturers, since it is known to prevent blocking of copolymers (Xu et al. 2005).

Several technologies for phytate removal from cereals and grains have been developed, mostly in order to increase the nutritional value and the bioavailability of nutrients linked to phytate. Mechanical processes, such as milling, have been reported as efficient phytate removal techniques in germ-rich grains, such as corn, in which 89% of phytate is found in the germ portion (Yi et al. 1996). In wheat, rye and rice, phytate is concentrated in the outer layers, and hence normal milling has been reported to remove most of phytate from these cereals (Harland and Oberleas 1987). The industrial process for phytate manufacturing consists on acid extraction from rice bran or cereals, followed by a chemical precipitation step. Strong acids are needed to dissolve phytate in traditional phytate extraction (Canan et al. 2011). If phytate were recovered from the end products, like DDGS, *via* the industrial process, the low pH of the possible product would likely make this unviable as feeding material.

Despite of the environmental problems faced with phytate in the corn ethanol industry, this molecule is often seen as a high-value chemical in the market. The process described in this article introduces a series of anion exchange resins to extract phytate phosphorus from corn-to-ethanol TS. The application of ion

exchange resins to extract phytate from ethanol coproducts has been evaluated by He et al. (2017). This chapter addresses a comprehensive understanding of other anion exchange resins, and how the extraction process could be added as a retrofit addendum to a corn-to-ethanol dry-grind plant.

## 5.2. Experimental

### 5.2.1. Sample collection

Samples of WS, TS, WDGS, CDS, and DDGS were obtained from a large scale dry-grind corn ethanol plant located in the state of Iowa, USA. Samples were kept frozen at -20 ºC prior to use.

### 5.2.2. Resin selection

A set of 6 ion exchange resins was selected as shown in Table 1, based on the previous studies that have reported the capacity of these anion exchange resins to adsorb phosphorus compounds (Blaney et al. 2007; Graf et al. 1987; Phillippy et al. 1987; Schwartz et al. 1972; Sharpley et al. 1984). SEM characterization was performed using Hitachi S3500N Variable Pressure Scanning Electron Microscope (SEM) to understand the physical characteristics of ion exchange resins (Meenakshi and Viswanathan 2007). SEM images were processed using KLONK Image Measurement software version 16.1.1.4. Images were processed and converted to a 3 dimensional profile, and pore distribution was estimated using the area measurement tool KLONK Image Measurement. Calculated porosity was then estimated by dividing the pore area ($\mu m^2$) by the resin bead volume, assuming a perfect sphere with the diameter as the average diameter provided by the resin manufacturer ($\mu m^3$).

Table 5.1: Physicochemical characteristics of the anion exchange resins

| Resin | IRA 93 | IRA 68 | IRA 900 | IRA 400 | IRA 402 | AG 1-X8 |
|---|---|---|---|---|---|---|
| Manufacturer | Dow/Rohm & Haas | Dow/Rohm & Haas | Dow/Rohm & Haas | Dow/Rohm & Haas | Dow/Rohm & Haas | BioRad |
| CAS Number | 9050-97-9 | 9056-59-1 | 9050-97-9 | 9002-24-8 | 52439-77-7 | 60177-39-1 |
| Product type | Weakly basic, macroreticular | Weakly basic, macroreticular | Macroporous, type 1 resin | Strongly basic, type 1 | Strongly basic, type 1 | Strongly basic |
| Matrix material | Poly(styrene-divinylbenzene) | Polyacrylate (gel) | Poly(styrene-divinylbenzene) | Poly(styrene-divinylbenzene) (gel) | Poly(styrene-divinylbenzene) (gel) | Poly(styrene-divinylbenzene) |
| Functional Group | $-N(R)_2H$ | $-N(R)_2H$ | $-N^+(CH_3)_3$ | $-N^+(R)_3$ | $-N^+(CH_3)_3$ | $-N^+(R)_3$ |
| Ionic form | Free Base | Free Base | $Cl^-$ | $Cl^-$ | $Cl^-$ | $Cl^-$ |
| Size (μm) | 400-700 | 500-750 | 650-820 | 600-750 | 600-750 | 106–180 |
| Moisture (%) | 48-58 | 50 - 60% | 58-64 | 40-47 | 49-60 | 39-45 |
| Calculated porosity (% $\mu m^{-1}$) | 1.603 | 3.099 | 6.539 | 4.458 | 2.235 | 1.216 |
| SEM Profile |  |  |  |  |  |  |

### 5.2.3. Ion exchange experiments

For batch experiments, 0.04 g of resin were weighed and added to 50 mL capped vials containing 40 mL of TS. The low amount of resin to TS ratio has been used to provide experimental situations in which the resin to be the limiting factor and reach the saturated capacity of these resins. Based on the resin capacity described by the manufacturers, and on the proximate anion analysis in TS, it is assumed all resin beads are in their full adsorption capacity after batch experiments.

For the evaluation of desorption capacity, the resin beads were removed through filtration, washed with distilled water to remove any solid aggregates, and added

to various eluent solutions at different concentrations. Different types of eluents (NaOH, NaCl, $NaHCO_3$, HCl, and $NH_4OH$), at 1 mol $L^{-1}$ concentration were tested to evaluate the desorption capacity in all the six resins. These eluents were intended to elute the P anions from the resin beads. Selection of the most effective desorbing agent was considered based on the cost-effectiveness and efficiency of desorbing PP from the resin into the eluent for final product recovery.

For breakthrough experiments, a chromatographic glass column (1.0 cm x 30 cm) was used as support to the loads of IRA-900. 3 mL of resin beads were added, and TS was fed using a peristaltic pump. For the desorption experiments, desorption agent solution was loaded sequentially at an increment of 3 mL, corresponding to 1 bed volume.

Adsorption capacity (AC), also referred as resin equilibrium capacity, $q_e$, for a species i (PP, RP, TP) was estimated as described in eq. 1.

$$AC_i = \frac{(C_{i,0} - C_{i,e})V}{Q_r} \quad (1)$$

In which $C_{i,0}$ is the concentration of species i at time 0 (mg $L^{-1}$), $C_{i,e}$ is the concentration of species i at the equilibrium time (mg $L^{-1}$), V is the control volume (L), and $Q_r$ is the total resin weight at time 0.

Desorption efficiency (DE) for a species i is described in eq. 2.

$$DE_i = \frac{(C_{i,d})V}{Q_r AC_i} \quad (2)$$

In which, $C_{i,d}$ is the concentration of species i on the liquid phase after desorption.

### 5.2.4. Kinetics and Isotherm plotting

The quantitative treatment of kinetic plotting of ion exchange was made combining the elements of film and mass transfer rate determining steps. It was determined experimentally by plotting the reduction in PP using IRA-900 resin in an agitated batch system. The isotherm determining experiment was made using increasing concentrations of IRA-900 resin on a fixed amount of TS. The evaluation of decrease in concentration of PP in TS and subsequent increase in adsorbed PP in IRA-900 was plotted.

### 5.2.5. Particle size analysis (PSA)

PSA is a measurement of the size distribution of individual particles in a sample. This method, widely used in soil science, distributes aggregates into discrete units by mechanical separation and the separation of particles according to size limits defined by sieves.(Gee and Or 2002) As TS has been reported the fraction with most P in a dry basis, PSA was conducted to evaluate weight fractions and P distribution on TS using filters with pore size of 5, 3, 1.2, 0.7, 0.45, 0.22, 0.05, and 0.025 µm.

### 5.2.6. P analysis

Total P (TP) and Reactive P (RP) were determined using P-ash method, in which digested samples were analyzed with a colorimetric assay of P in the digest. Samples were first dried at 105 $^{o}$C until a constant dry weight, and then ashed at 550 $^{o}$C. Concentrated $HNO_3$ (7 mol $L^{-1}$) is used to dissolve the inorganic ash

residue (Taussky and Shorr 1953). Spectrophotometric assays were then performed with the molybdenum blue method (Murphy and Riley 1962).

An enzymatic method based on De Boland et al. (1975) and McKie and McCleary (2016) was used for the determination of phytate P (PP), which is based on the principle that phytase hydrolyses phytic acid into lower inositol-phosphates and orthophosphate ($P_i$).

$$\text{phytic acid} + H_2O \xrightarrow{(phytase)} \textit{myo}\text{-inositol (phosphate)}_n + P_i$$

Alkaline phosphatase further hydrolyses *myo*-inositol $(\text{phosphate})_n$ producing *myo*-inositol and $P_i$.

$$\textit{myo}\text{-inositol (phosphate)}_n + H_2O \xrightarrow{(alkaline\ phosphatase)} \textit{myo}\text{-inositol (phosphate)}_n + P_i$$

The amount of $P_i$ formed in these reactions was quantified colorimetrically through the molybdenum blue method (Murphy and Riley 1962). $^{31}$P-NMR analysis was used to analyze the inositol-phosphate distribution among different fractions of ethanol coproducts using the method described by Frølich et al. (1986).

### 5.2.7. Statistical analysis

All results were expressed as mean ± standard deviation based on a triplicate analysis. The least significant difference (LSD) method was used to compare P content of corn and stillage processing streams. LSD method is a two-step testing procedure for comparison of pairs of several treatment groups. In the first step, a global test is performed for the null hypothesis that the expected means of all

treatments, which is the different fractions of P in the coproducts, are equal. If this global null hypothesis can be rejected at the level of significance of 5% (to be used in this study), the second step analyzes all pairwise comparisons at the same level of significance (Meier 2006).

## 5.3. Results and Discussion

### 5.3.1. Particle size analysis and IP distribution among different ethanol coproducts

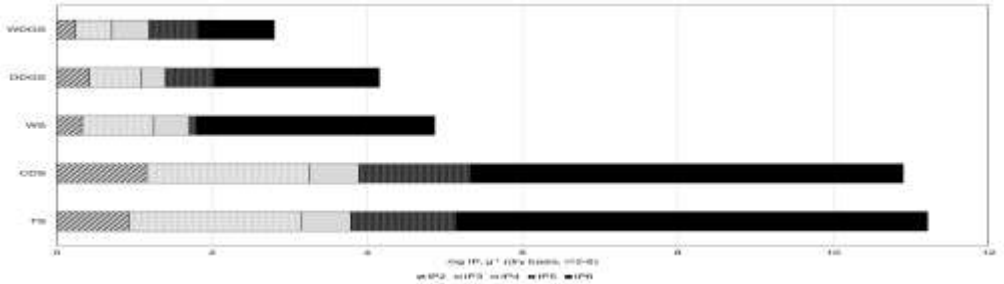


Figure 5.1.: Inositol phosphate distribution among ethanol coproducts

Among the ethanol coproducts analyzed, levels of IP fractions as $IP_6$ were greater than 50% in all the samples. Of the samples analyzed, about 54% of the total IP fractions were in the form of $IP_6$ in TS (Figure 1). Since the adsorption phenomena studied occurs between P groups and the resin, the most substituted fractions yield better adsorption. Phytate was found most concentrated in the small-size range of particles in TS, indicating that it was possible to solubilize or aggregate these small particles (Figure 2). Selecting the appropriate size range of particles is crucial in order to process only the most concentrated fraction, which will allow the most efficient adsorption operation.

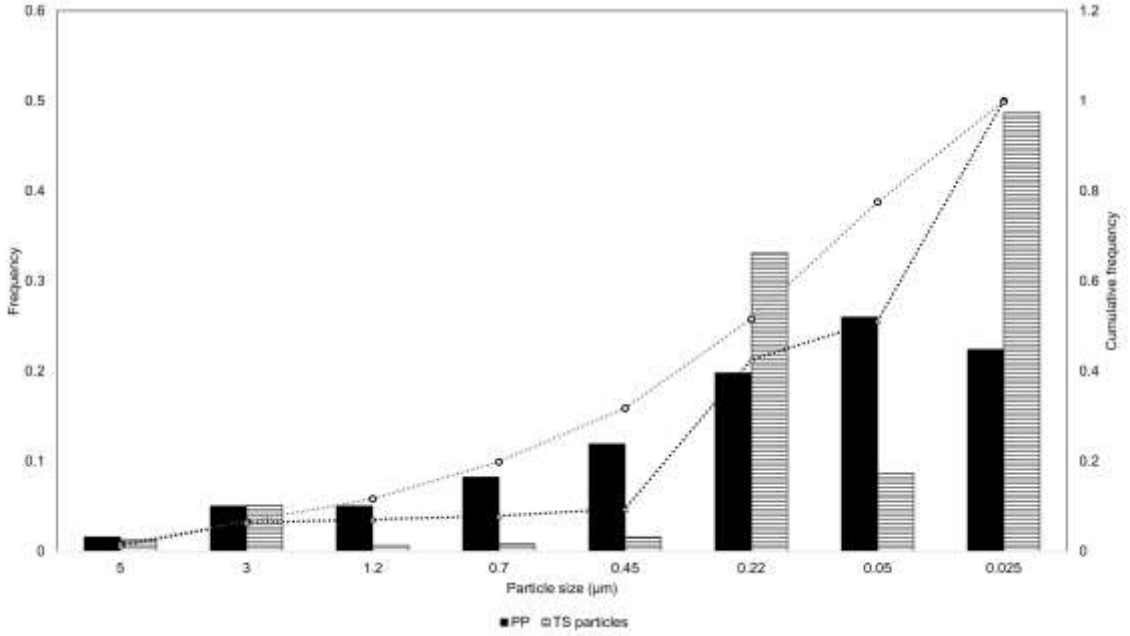
Naidu et al. (2007) described solubles in ethanol coproducts as smaller than 2 mm in diameter. These are often not removed by centrifuges in the WS separation. As

described by other reports(Alkan-Ozkaynak et al. 2010), TS is the fraction which contains the greatest concentration of IP6. Cromwell (Cromwell 1979) was among the first authors to report the capability of yeast in hydrolyzing organic P, especially phytate. Phillippy et al. (1987) evaluated the potential of industrial yeasts in hydrolyzing inositol IP6 during ethanol fermentation, and reported that the strains were able to produce phytases. These enzymes are able to degrade phytate to forms of inositol mono- ($IP_1$), bis- ($IP_2$), tris- ($IP_3$), tetra- ($IP_4$), and pentakisphosphates ($IP_5$), as well as to inorganic phosphate. However, according to Liu and Han (2011), due to fermentation conditions often employed, about 40% to 50% of P in the fermentation mash usually remains as IP6. TS characterization conducted by Alkan-Ozkaynak et al. (2010) presented higher total solids content (7%) than the one used in this study (5.3%), and a significant difference in solids smaller than 0.45 µm (65% for Alkan-Ozkaynak et al. (2010) and approximately

90% for this study). This difference may be due to a number of factors, including fluctuations in the ethanol process conditions among different plants.

Figure 5.2: Particle size and PP distribution in TS – Cumulative frequency: ●PP ▲TS particles

### 5.3.2. Selection of resin for phytate adsorption




Comparison of five macroporous resins (IRA series) and the fine resins (AG-1 series) for the phytate adsorption capacity, selectivity, purity after desorption, and low maintenance requirements are shown in Table 2. The liquid fraction of the TS, which is the phytate-rich ethanol co-product is evaluated as the raw material for the ion exchange process. TS also has significant presence of phosphate and other types of P-based groups, which were also adsorbed onto the resin. The desirable characteristics of the most effective resin are those with the capability to adsorb most of the PP and the least amount of RP, thus determining the resin

selectivity. The adsorption capacity of the different types of resins, IRA-93, IRA-68, IRA-900, IRA-400, IRA-402 and AG 1-X8 is shown in Table 2. IRA-400 (0.128 g PP $g^{-1}_{resin}$) and IRA-900 (0.128 g PP $g^{-1}_{resin}$) resins adsorbed the most PP per mass of resin, but IRA-900 (0.73 PP/TP) showed more selectivity compared to the IRA-400.

Table 5.2.: Adsorption capacity (AC) of different P forms

| | PP (g $g^{-1}$) | RP (g $g^{-1}$) | TP (g $g^{-1}$) | PP/TP |
|---|---|---|---|---|
| IRA 93 | 0.10 | 0.05 | 0.15 | 0.65 |
| IRA 68 | 0.10 | 0.06 | 0.16 | 0.60 |
| IRA 400 | 0.13 | 0.06 | 0.19 | 0.66 |
| IRA 900 | 0.13 | 0.07 | 0.17 | 0.73 |
| IRA 402 | 0.12 | 0.06 | 0.17 | 0.70 |
| AG 1-X8 | 0.10 | 0.07 | 0.18 | 0.57 |

The selection of a resin for a chromatographic separation is the most important step in the overall process development (Rathore 2001) . The key criterion to be used in resin selection is selectivity towards the preferred analyte. The mechanism of ion exchange separation is related to the differential ionic interactions between the ligands on the resin to the charged components in the bulk solution. Since most of all these resins are of similar chemical composition (divinylbenzene matrix with secondary or tertiary amine exchange groups), it is believed that surface properties are responsible for the differences in adsorption efficiency among the different resins. On a process development point of view, rigid resins, such as IRA 900,

would be preferable for harsh conditions (low pH and large columns) when compared to gel-type resins (Jones and Carta 1993).

IRA-93 (macroreticular type I strong alkali resin) and IRA-68 (microreticular type I strong alkali resin) were reported on a study targeting the recovery of organic acids and phosphate from potato starch factory wastes (Schwartz et al. 1972). IRA-900 (macroreticular type I strong alkali resin) has been used to extract phosphate from water and wastewater sources (Blaney et al. 2007). IRA-400 has been evaluated as a substitute for a quicker and easier application of labile, organic, and sorbed P in soil samples(Sharpley et al. 1984). IRA-402 has been used to separate reactive phosphorus in lake water samples(Downes and Paerl 1978). AG 1-X8 resin has been used by to purify phytate extracts prior to HPLC analysis(Graf and Dintzis 1982). Among the resins, IRA-900 was the one with the highest calculated porosity (6.53 $\mu m^{-1}$ %) but there are no reports on utilizing IRA-900 to extract phytate from liquid solutions. Though the surface properties of anion exchange resins on the adsorption properties of phytate play an important role, it can be hypothesized that the dense electronegative cloud of PP would perform better on a highly porous material, when compared to a resin with lower degree of porosity (AG 1-X8, for example, with a porosity of 1.2 $\mu m^{-1}$ %).

### 5.3.3. Selection of desorbing agent and concentration

PP desorption was found to be more effective and selective with NaCl in all resins tested (Table 3), which was selected as the desorbing agent for further studies.

NaCl (1 mol $L^{-1}$) was the most selective and most efficient phosphorus desorbing agent in all resins. DE was as high as 0.80 for IRA-93. Though $Cl^-$ may be a suitable regeneration anion for all resins, results for HCl (1 mol $L^{-1}$) are significantly lower when compared to NaCl at the similar concentration. DE for TP in all cases was lower, except for IRA-400, which achieve DE of 0.53 in both conditions for TP, but with significant lower DE for PP (0.65 for NaCl and 0.23 for HCl).

NaCl has been selected as desorption agent in previous reports in phytate purification using ion exchange chromatography. NaCl was used to selectively desorb phytate over inorganic phosphate using AG 1-X8, using an acid extract of phytate from cereals as bulk solution (Latta and Eskin 1980). Helfferich (1962) (Helfferich 1962) described NaCl as being a preferable desorbing agent, which could even be used as regeneration agent in some cases, due to the low-toxic potential and low cost involved. DE is a function of the concentration of desorbing agent and the amount of PP adsorbed by the resin. Since IRA-900 shows better PP adsorption and selectivity, the concentration of the NaCl was varied from 1 to 5 mol $L^{-1}$ (Table 4) to find the optimum concentration for higher desorption efficiency. With 2 mol $L^{-1}$ of NaCl, 100% desorption of PP (DE = 1.0) was achieved with the IRA-900 resin.

Table 5.3.: Desorption efficiency (DE) of different desorbing solutions at 1 mol $L^{-1}$

| | | NaOH | $NaHCO_3$ | $NH_4OH$ | NaCl | HCl |
|---|---|---|---|---|---|---|
| | PP | 0.22 | 0.29 | 0.58 | 0.80 | 0.02 |
| IRA 93 | RP | 0.06 | 0.06 | 0.10 | 0.04 | 0.02 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | TP | 0.42 | 0.42 | 0.21 | 0.69 | 0.55 |
| | PP | 0.26 | 0.53 | 0.04 | 0.77 | 0.00 |
| IRA 68 | RP | 0.01 | 0.03 | 0.29 | 0.03 | 0.26 |
| | TP | 0.24 | 0.90 | 0.73 | 0.73 | 0.44 |
| | PP | 0.09 | 0.03 | 0.02 | 0.65 | 0.23 |
| IRA 400 | RP | 0.03 | 0.42 | 0.06 | 0.02 | 0.02 |
| | TP | 0.05 | 0.04 | 0.02 | 0.53 | 0.53 |
| | PP | 0.11 | 0.07 | 0.02 | 0.30 | 0.01 |
| IRA 900 | RP | 0.02 | 0.04 | 0.01 | 0.04 | 0.22 |
| | TP | 0.42 | 0.18 | 0.01 | 0.27 | 0.10 |
| | PP | 0.11 | 0.07 | 0.02 | 0.29 | 0.01 |
| IRA 402 | RP | 0.02 | 0.04 | 0.01 | 0.04 | 0.21 |
| | TP | 0.42 | 0.18 | 0.01 | 0.27 | 0.01 |
| | PP | 0.03 | 0.01 | 0.01 | 0.01 | 0.01 |
| AG 1-X8 | RP | 0.01 | 0.29 | 0.03 | 0.29 | 0.19 |
| | TP | 0.16 | 0.05 | 0.06 | 0.02 | 0.41 |

Table 5.4.: Desorption efficiency comparing different NaCl concentrations using IRA 900 resin

| NaCl (mol $L^{-1}$) | TP | RP | PP |
|---|---|---|---|
| 1.0 | 0.53 | 0.01 | 0.65 |

| | | | |
|---|---|---|---|
| 1.5 | 0.69 | 0.12 | 0.86 |
| 2.0 | 0.83 | 0.13 | 1.00 |
| 3.0 | 0.79 | 0.19 | 0.92 |
| 4.0 | 0.85 | 0.13 | 0.93 |
| 5.0 | 0.88 | 0.15 | 0.99 |

### 5.3.4. Resin regeneration capacity

The concentration of RP, TP, and PP in the batch regeneration experiments are presented in Figure 3. The P concentration in TS decreased in all experiments after the contact time, as expected, resulting in resin concentrations of TP ranging from 140 to 190 mg $g^{-1}$ on IRA-900. The experimental conditions applied on the regeneration presented a level of P recalcitrance on IRA-402 resin, decreasing its TP adsorption equilibrium capacity from 160 mg $g^{-1}$ to approximately 120 mg $g^{-1}$ after its first regeneration. Similar recalcitrance profile was observed for IRA-400 resin, decreasing its TP adsorption capacity from approximately 140 to 170 mg $g^{-1}$ after 4 regeneration cycles. P recalcitrance on a separation system for continuous use is strongly undesired, since it would systematically decrease the performance of the adsorption and desorption system, thus affecting the phytate recovery process. IRA-900 and IRA-93 resins were the resins with the least recalcitrance among the industrial resins tested, posing difference in adsorption capacities of Pspecies. IRA-900 performance for PP adsorption was greater on all the regeneration cycles when compared to IRA-93, with adsorption equilibrium capacities ranging from 95 to 120 mg $g^{-1}$. The optimal resin should have an

extended lifespan, which decreases the overall operating costs and increases technical feasibility of the separation process.

The proposed mechanism for chemical regeneration of the saturated resin is described as follows: i) P anions, simplified written as R-($H_xPO_4^{3-x}$), and other anions not evaluated in the current study, including $NO^{3-}$, $NO^{2-}$, $SO_4^{2-}$ are adsorbed onto the resin, replacing the free base anion ($OH^-$) or the $Cl^-$ anion present in the resin for the ion exchanged species; (ii) The $Cl^-$ present in the NaCl solution, described on this article as desorbing agent, replaces the species adsorbed onto the resin, releasing the target anions to the bulk saline solution; (iii) NaOH is used to regenerate the resin, replacing the anions not replaced by $Cl^-$ of the desorbing agent to free base anions. Liu et al. (2016) described a similar regeneration system for P anions removal from a bulk solution, and experienced similar recalcitrance of some anionic species. Despite the harsh conditions used for regeneration (NaOH at a concentration 150 g $L^{-1}$ with a loading rate of 4%), it is believed that the third step of the proposed mechanism is the limiting step in the regeneration, on a similar fashion experienced by Liu et al. (2016).

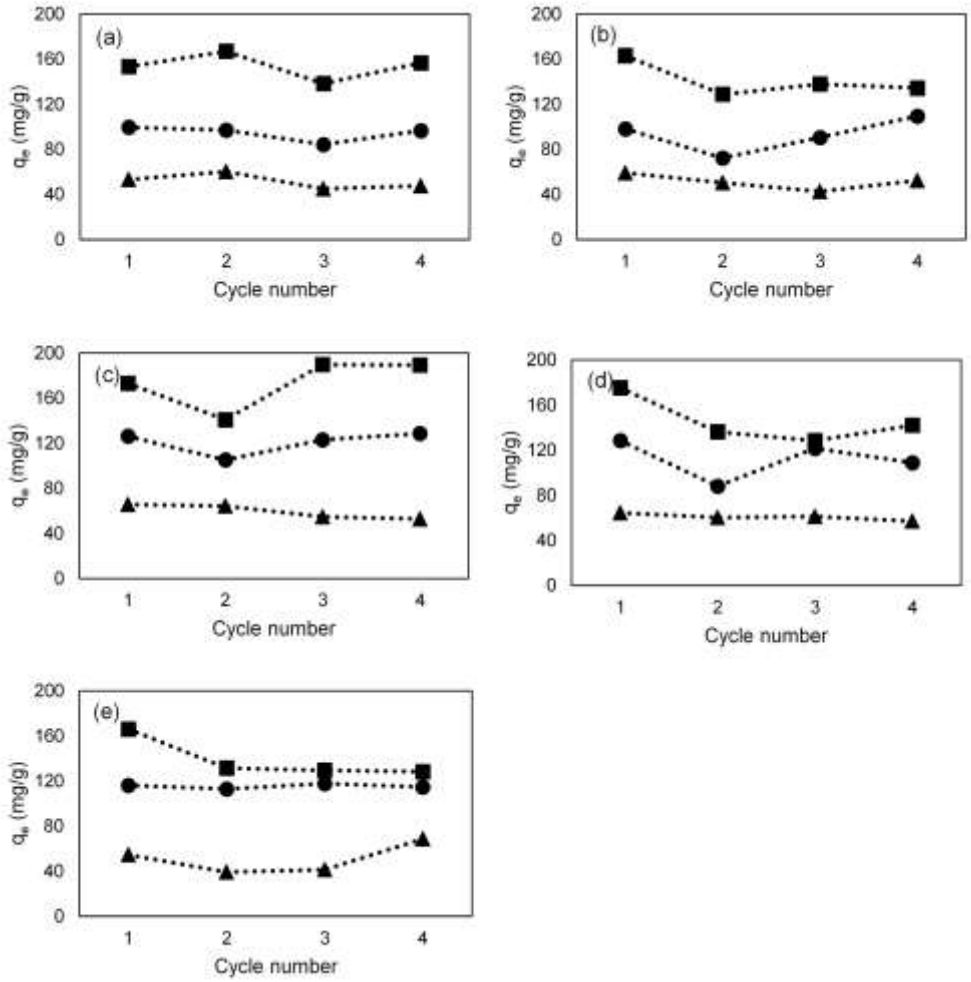



Figure 5.3.: Regeneration profile for adsorption among different resins (a) IRA 93 (b) IRA 68 (c) IRA 900 (d) IRA 400 (e) IRA 402 - ●PP ▲RP ■ TP

### 5.3.5. Isotherm, kinetics and breakthrough curves

The adsorption isotherm shown in Figure 5.4a represented a favorable profile indicating that even at low concentrations, the resin could be loaded with high amount of PP. The adsorption isotherms predicted the maximum adsorption capacity of each resin, which were close to those obtained experimentally. They were expressed in amount of substrate adsorbed in equilibrium onto the resin ($q_e$) *vs.* the residual substrate in solution in equilibrium ($C_e$). The kinetics of adsorption also showed that phytate adsorption was a moderately fast process, achieving 100% of adsorption at 2 h of contact time (Figure 4b). Breakthrough curve evaluated the resin bed's band of exhaustion. It related the concentration history, reported in this article as $C/C_0$ over a volume axis. Results indicated a maximum loading amount of 13 bed volumes prior to significant leakage ($C/C_0$>0.05) of PP (Figure 5.5).

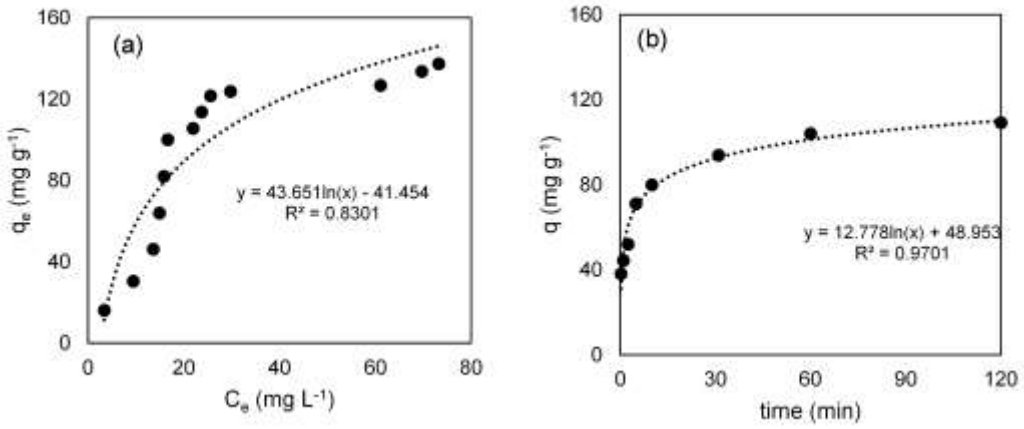



Figure 5.4.: (a) PP Isotherm (b) Kinetics of PP adsorption

Most reports in the literature use anion exchange resin to purify PP in order to analytically measure its content in cereals (Latta and Eskin 1980) and foods (Harland and Oberleas 1987). Previous chromatographic separations of phytate from solutions containing RP reported in the literature achieved separation of phytate within 30 minutes(Latta and Eskin 1980). Vaintraub and Lapteva (1988) conducted experiments to separate phytate from other anions and operated their system in a column without control of residence time. In both cases, the resin to solution ratio allowed a high adsorption rate. No results have been found in the literature, however, in terms of analyzing the maximum capacity, or detailing its column operation and kinetics performance on regards to phytate extraction using ion exchange resins.

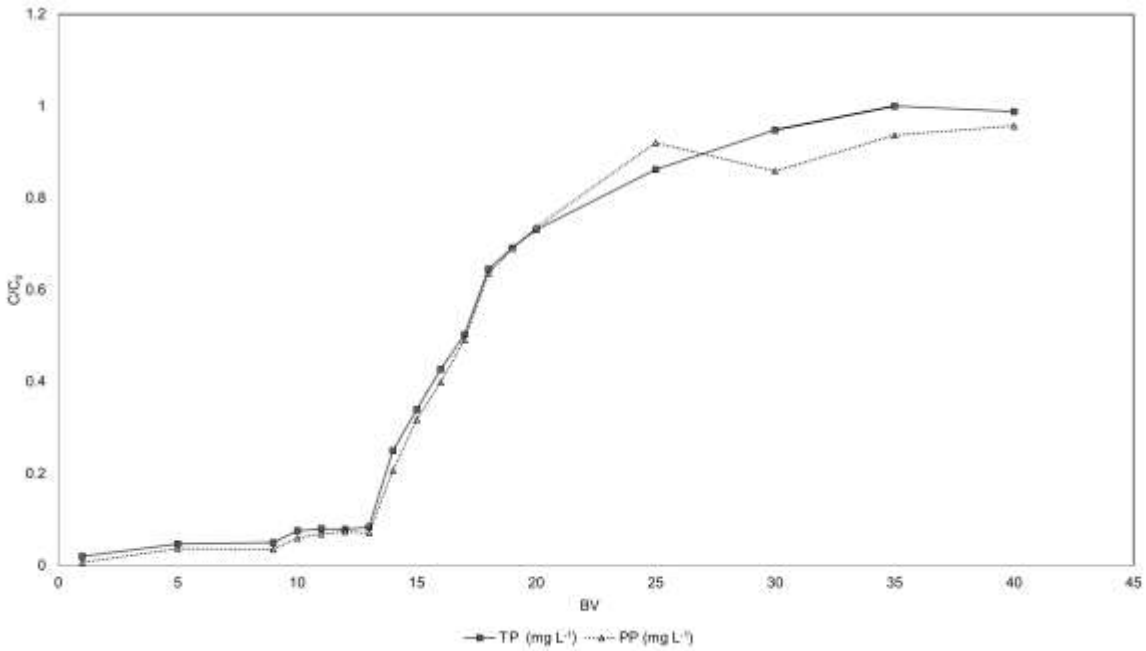



Figure 5.5.: Breakthrough profile on IRA-900-resin packed column

### 5.3.6. Elution profile and effect of resin to desorbing agent ratio

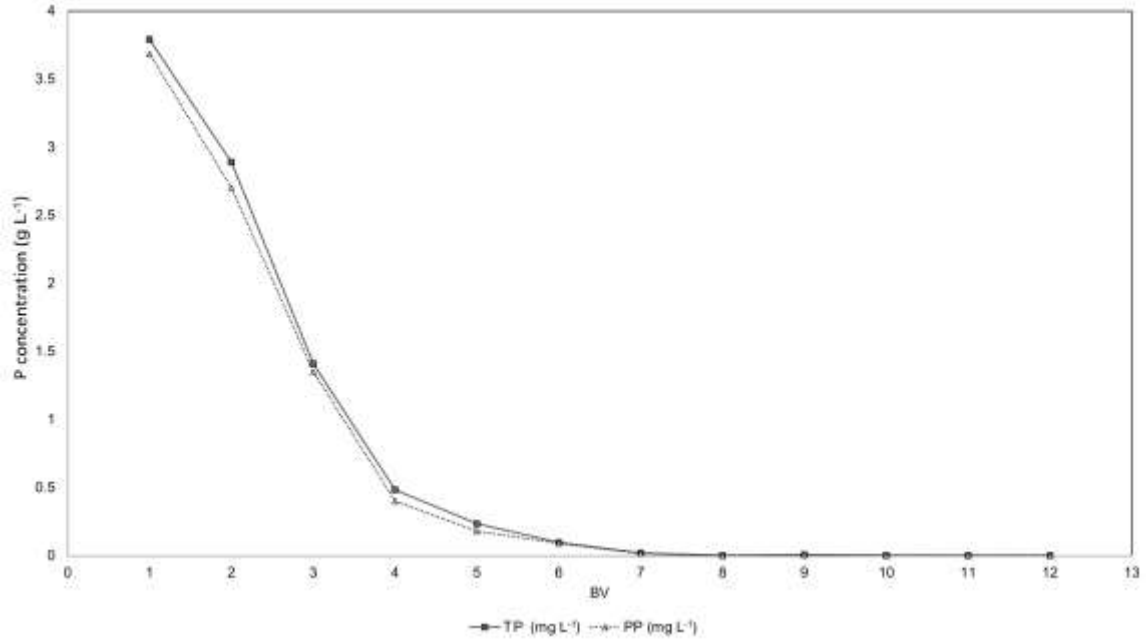



Figure 5.6.: PP elution curve

NaCl (2 mol $L^{-1}$) was used as desorbing agent on column experiments. The high efficiency predicted by batch desorption experiments is confirmed in column desorption. Within 5 bed volumes of NaCl addition, eluents with high concentrations of phytate are obtained (Figure 6). 7 bed volumes of NaCl solution are sufficient to completely remove all P in column. Since there are no reports in the literature on previous detailed analysis of phytate adsorption and desorption using similar technology, comparison with previous results is not possible. A batch system can also be used for desorption. In batch conditions, an increase in resin to desorbing agent ratio leads to a subsequent increase of desorbed PP. A concentration of about 20 g $L^{-1}$ was obtained with a resin to NaCl solution ratio of 100 weight/volume %. However, results indicated that after resin to NaCl solution

ratios greater than 30% did not promote 100% of desorption under the studied conditions (Figure 7).

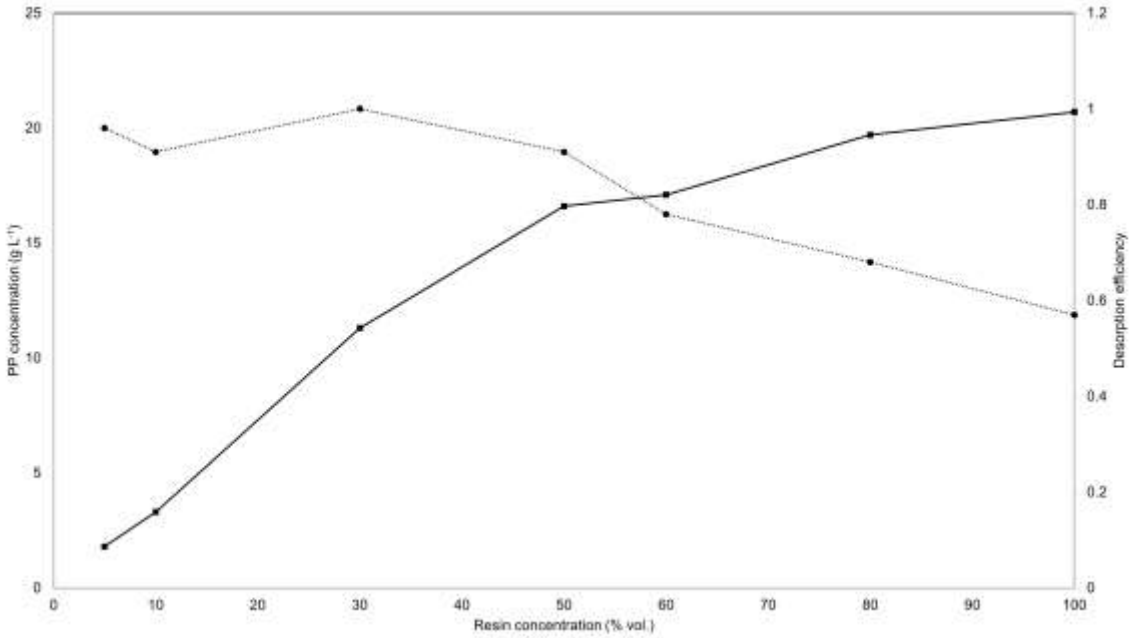



Figure 5.7.: ■ Effect of resin loading rate (weight/volume %) on PP concentration; ● Efficiency of resin loading rate (weight %) on PP desorption

### 5.3.7. Retrofit process development

In preparative chromatography, a variety of parameters impact the production rate and recovery of a desired compound, including the stationary and mobile phases, the particle size of the stationary phase, the feed loading, column design, and flow rate. The process developed on this article selected the most effective mode of interaction between a mobile phase (TS), and a solid phase (Amberlite$^{TM}$ IRA-900 anion exchange resin), resulting in a relatively simple packed bed anion exchange resin with high specificity for the compound of interest – phytate. The experimental conditions applied in this study indicate an extraction efficiency of 429.5 mg PP L$^{-1}$ TS on a column operation. Assuming all the PP extracted and purified as IP6, a

molar conversion can be estimated by a factor of 3.552 (phytic acid molar mass/TP molar mass in IP6: 660.04 g mol $^{-1}$/ 6 * 30.97 g mol $^{-1}$). Thus, a liter of TS would be feedstock for extracting 1.52 g of phytic acid, measured as $IP_6$. Phytic acid has a commercial value greater than all the commercial products generated by a dry-grind ethanol plant, at a market price at around 50 U.S. dollars per kg (pharmaceutical supplement form, as of January 2017). Traditional distiller's grains are often sold at no more than 200 U.S. dollars per metric ton; therefore, phytic acid would potentially enhance the profitability of a dry-grind ethanol plant. The process on figure 5.8 suggests the modifications to the dry-grind corn-to-ethanol industry. Despite still found at dilute concentrations, the removal of phytic acid from ethanol coproduct streams can provide significant economic gains for the corn-to-ethanol industry, and can indirectly play a role in other operating costs in the downstream processing. The presence of phytic acid in distillery downstream processing has been described as a source of fouling, therefore, the extraction of such compound would potentially decrease the maintenance costs related to anti-fouling agents applied.

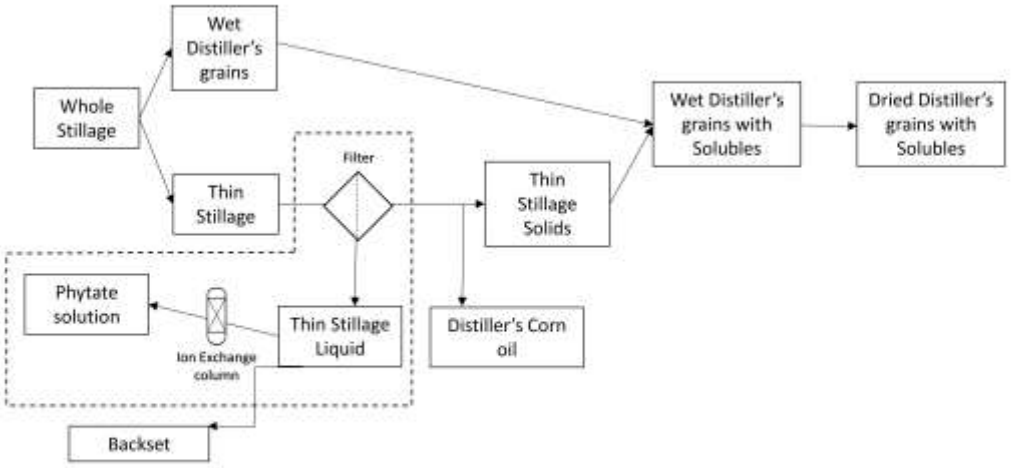



Figure 5.8.: Proposed retrofit process to extract phytate from ethanol coproducts (additional unit operations on retrofit are within the dashed line)

### 5.4. Conclusions

The results summarized in this article advance the understanding of P distribution in corn-to-ethanol coproducts, and evaluate a technically sound process to extract phytate from the ethanol downstream operations. Amberlite™ IRA-900 has been selected as the most selective and effective resin for phytate extraction under the conditions tested, using industrial corn-to-ethanol TS as raw material, due to its low recalcitrance for P species, and high capacity. This process will not only to provide an alternative for another source of income to corn-to-ethanol plants, but also an addendum to analytical methods for phytate which use purification steps via ion exchange chromatography. The resin provided in this study is of lower cost than AG 1-series resin commonly cited for such application. The future direction

for a deeper understanding of phytate extraction from ethanol coproducts includes the molecular mechanism description of inositol phosphates as different fractions on the ion exchange resin, knowing that each different inositol phosphate fraction has a different electron density, which directly impact the ion exchange phenomena, and the scale-up analysis of the proposed extraction process.

# CHAPTER 6: VOLUMETRIC SCALE-UP OF A PACKED-BED ION EXCHANGE SYSTEM TO EXTRACT PHYTATE FROM THIN STILLAGE

## 6.1. Background

The use of preparative chromatography is one of the dominant purification techniques in the production of biological compounds, such as proteins, and pharmaceutical-grade chemicals. The conceptual complexity of a purely theoretical approach to preparative chromatography, however, imposes innumerous challenges to a purely theoretical approach to a complete understanding of biotechnological separations. For ion exchange chromatography, for example, Rathore and Velayudhan (2002) described a scale-up approach being preferable to run optimization on a small-scale followed by simplifications on a larger process development. A series of parameters, including the choice of mobile and stationary phases, particle size and particle size distribution, the column dimensions, the flow rate, and the feed loading properties, affect the production rate and recovery obtained in a chromatographic operation.

Chromatographic column separations depend on a variety of design and operating factors. A successful scale-up usually involves several factors being fixed on a small and a large scale, such as those involved kinetic factors (particle size, pore size, temperature, mobile phase, and ligand chemistry, for example), and dynamic variables (bed height, flow velocity, and packing density). The most common simplification in understanding chromatographic processes is expressed as the van Deemter equation.

$$H = A + \frac{B}{u} + Cu$$

The van Deemter equations relates the linear flow velocity (u), the plate height present at a chromatographic column (H), and constants for each system considered (A, B, and C).

### 6.2. Isotherm development

The understanding of an isotherm that regulates an adsorption system is fundamental for a column design. An isotherm will describe the adsorption profile of a column for a specific compound, and how easily it is saturated. A variety of mathematical models have been described to predict the breakthrough profiles of a column adsorption system (Xu et al. 2013). Xu et al. (2013)described the modeling of a liquid-solid adsorption system being regulated by four basic steps: (i) liquid mass transfer, (ii) interface diffusion between the liquid phase and the exterior surface of the adsorbent, (iii) intrapellet mass transfer, and (iv) the adsorption-desorption reaction.

(i) Liquid mass transfer is regulated by convective mass transfer and molecular diffusion, in which species can move in axial directions. On a macroscopic scale, the simplest equation describing movements of species in adsorption systems could be written as:

$$\varepsilon \frac{\partial C}{\partial t} + u \frac{\partial C}{\partial z} + (1 - \varepsilon)\rho_a \frac{\partial q}{\partial t} = D_z \frac{\partial^2 C}{\partial z^2}$$

For which the initial and boundary conditions are as follows:

$$t = 0 \rightarrow C(z, t) = 0$$

$$t = 0 \rightarrow q(z,t) = 0$$

$$z = 0 \rightarrow C(0,0) = 0$$

$$C(0, z > 0) = C_F$$

$$z = H \rightarrow \frac{\partial C}{\partial z} = 0$$

If systems with negligible axial dispersions are considered, the equation X turns to:

$$\varepsilon \frac{\partial C}{\partial t} + u \frac{\partial C}{\partial z} + (1 - \varepsilon)\rho_a \frac{\partial q}{\partial t} = 0$$

Thus, changing the initial and boundary conditions to:

$$t = 0 \rightarrow C(z,t) = 0$$

$$z = 0 \rightarrow C(z,t) = C_F + \frac{D_z \varepsilon}{u} \frac{\partial C}{\partial z}$$

$$C(0, z > 0) = C_F$$

$$z = H \rightarrow \frac{\partial C}{\partial z} = 0$$

The assumptions behind the equation above are that the process is isothermal with no chemical reaction altering the composition of the species involved in the adsorption-desorption process, and that the packing material is uniform with perfect spheres that provide a homogeneous flow with zero radial gradient.

(ii) Film diffusion is regulated by the concentration gradient located at the interface between the exterior surface of an adsorbent and the bulk solution. Tien (1994) described the film diffusion step as the predominant factor as the first step of adsorption, controlling overall uptake to some extent and even controlling the

rate controlling step in some observations. The flux film diffusion can be expressed as a modification of Fick's law of diffusion, and is expressed as:

$$\frac{dq}{dt} = J_F = k_F a(C - C_s)$$

From the equation above, it can observed that an increase in the flow rate will decrease film thickness and resistance. The inverse is also true, on a sense that an increase in film thickness is caused by effects that can lower the flow rate, such as smaller adsorbents, and higher packing density.

(iii, iv) Intrapellet diffusion and chemical reaction: Surface diffusion and pore diffusion occur in parallel with Knudsen-type diffusion and chemical reactions. Knudsen diffusion is mostly significant in systems in which the diameter of the adsorbate being slightly larger than the diameter of the adsorbing species. Intrapellet diffusion is regulated by a series of complex equations, and often induce large errors when applied to experimental systems. Due to the heterogeneity of all adsorption systems, especially in the case of this work, in chromatographic separations, the modeling of purely intrapellet diffusion and chemical reaction can yield to uncertain results. As recalled by Xu et al. (2013), often times mathematical models are used to simplify the conditions imposed by intrapellet diffusion and chemical reactions to macroscopic equilibrium relationships as isotherms.

Malek and Farooq (1996) suggested three fundamental means to derive and formulate an isotherm: (i) dynamic equilibrium between adsorption and desorption, (ii) thermodynamic equilibrium between phases and species, and (iii)

adsorption potential theory. Morgenstern (2004) reviewed the means to derive isotherm modelling, and provides a discussion of the most common models. The models to be discussed herein are: Langmuir, Freundlich, and the basis for multi-component adsorption systems.

Langmuir isotherm assumes that the adsorption process takes place within a single layer onto the adsorbent, formed by homogeneous sites and that the heat of adsorption does not change with coverage. The most used model for isotherm modeling, Langmuir, also assumes that the adsorption takes place when a free adsorbate species collides with an unoccupied adsorption site, and that each species has the same probability of colliding with each site. The general formula for Langmuir model is as follows:

$$q_e = \frac{q_m b C_e}{1 + b C_e}$$

The constant b corresponds to the ratio of adsorption and desorption coefficients from the Langmuir kinetic models.

Freundlich isotherm have fewer limitations when compared to Langmuir, on a sense that it considers both homogeneous and heterogeneous surfaces, and no distinction between chemical and physical adsorption. It was first expressed due to the assumption that the differential heat of adsorption being related to the difference between the initial heat on the surface and the natural logarithm of the covered area, corrected by a constant factor. Freundlich isotherm is expressed as:

$$q_e = K C_e^{\frac{1}{n}}$$

For multi-component systems, i.e., those considering that more than one species is involved in the adsorption phenomena, the equilibrium relationship of any component may not fit the single-component isotherms, since competitive adsorption occurs in the overall process. The ideal adsorbed solution theory, according to Xu et al. (2013), can be applied to derive a general equation for multi-component adsorption isotherms. Myers and Prausnitz (1965) derived a model derived from the Gibbs adsorption formulation, which reads as:

$$\pi_i(q_i) = \frac{RT}{a}\int_0^{-q_i^*} \frac{d}{d}\frac{\log C_i}{\log q_i} dq_i$$

To which, the series of conditions on equations below define the degrees of freedom required to solve for each component:

$$C_i = z_i C^*$$

$$\frac{1}{q_T} = \sum_i^N \frac{z_i}{q_i}$$

$$q_i = z_i q$$

$$\sum_i^N z_i = 1$$

$$q_i = f(C_i)$$

A list of multi-component isotherms based on the work by Myers and Prausnitz (1965) is as follows:

(i) Multi-component Langmuir isotherm (Silva et al. 2010):

$$q_{e,i} = \frac{q_m b_i C_{e,i}}{1 + \sum_i^n b_i\, C_{e,i}}$$

(ii) Multi-component Langmuir-Freundlich isotherm (Ruthven 1984)

$$q_{e,i} = \frac{q_m b_i \left(C_{e,i}\right)^{k_i}}{1 + \sum_i^n b_i \left(C_{e,i}\right)^{k_i}}$$

(iii) Jain and Snoeyink's (1973) model assuming that the adsorptive capacity of species 1 is higher than species 2, and that the surplus is treated as a single-component adsorption

$$q_{e,1} = \frac{q_{m,2} b_1 C_{e,1}}{1 + b_1 C_{e,1} + b_2 C_{e,2}} + \frac{\left(q_{m,1} - q_{m,2}\right) b_1 C_{e,1}}{1 + b_1 C_{e,1}}$$

$$q_{e,2} = \frac{q_{m,2} b_2 C_{e,2}}{1 + b_1 C_{e,1} + b_2 C_{e,2}}$$

(iv) Chong and Volesky's (1995)model assuming that the formation of a bond between the adsorbent and the two species (as Ad-Sp1-Sp2)

$$q_{e,1} = \frac{q_m b_1 C_{e,1} \left[1 + \left(\frac{K'}{b_1}\right) C_{e,2}\right]}{1 + b_1 C_{e,1} + b_2 C_{e,2} + 2K C_{e,1} C_{e,2}}$$

$$q_{e,2} = \frac{q_m b_2 C_{e,2} \left[1 + \left(\frac{K'}{b_2}\right) C_{e,1}\right]}{1 + b_1 C_{e,1} + b_2 C_{e,2} + 2K'' C_{e,1} C_{e,2}}$$

(v) Wen-Bo et al. (2009) assumes that adsorption of species Sp1 and Sp2 are as follows: $n_1 Sp_1 + R_s \Leftrightarrow R_s Sp_{1n_1}$ and $n_2 Sp_2 + R_s \Leftrightarrow R_s Sp_{2n_2}$

$$q_1 = \frac{n_1 R_{sm} K_1 C_1^{n_1}}{1 + K_1 C_1^{n_1} + K_2 C_2^{n_2}}$$

$$q_2 = \frac{n_2 R_{sm} K_2 C_2^{n_2}}{1 + K_1 C_1^{n_1} + K_2 C_2^{n_2}}$$

In which:

$$K_i = \frac{[R_s M_{in_i}]}{[M_i]^{n_i}[R_s]}$$

### 6.3. The van Deemter model

The van Deemter model describes the mechanisms of band broadening in chromatographic separations for non-ideal separations, i.e., those that do not follow a linear isotherm. Van Deemter et al. (1956) described their rate theory development in systems in which non-ideality are caused by axial molecular diffusion, axial Eddy diffusion, and finiteness of transfer coefficient. For a system that follows the two rules of ideal chromatography, in which (i) the equilibrium concentrations are proportional in the two phases, and (ii) the exchange process is reversible according to purely its thermodynamic parameters, i.e., the equilibrium between particle and fluid is immediate, the mass transfer coefficient tends to positive infinity, and there are no diffusion effects within the system, the partition coefficient is related to the ratio of the amounts of those phases present in a column. For ideal chromatography, then, the separation of different solutes can be explained on arithmetic approximations. For systems in which non-linear isotherms are present, i.e., in systems in which the effect of nonlinearity of the isotherm cannot be neglected, usually comprehend conditions with fast mass transfer, and that axial diffusion can be neglected, according to DeVault (1943) – the first to report comprehensive studies on nonlinear ideal chromatographic

separations. DeVault (1943) described bands in nonlinear ideal chromatography as sharp-front and long-tailed.

For non-ideal chromatography and nonlinear isotherms, which comprehend most of the adsorption processes in mixture separations, Van Deemter et al. (1956) described a comprehensive cooperation of two theories – the rate theory and the plate theory. The plate theory describes the separation efficiency of a chromatographic column by the height equivalent to a theoretical plate (HETP, or simply, H). The rate theory provides all information on the influence of kinetic phenomena and lies its idealization within its acceptability at specific conditions, e.g. thin film cases. The application of Ketelle and Boyd (1947) and Boyd (1951) in which they analyzed the interpretation of a rate coefficient through a liquid film, were basis for Van Deemter et al. (1956) to further combine the conditions raised by Glueckauf (1955), who related plate height to particle size, particle diffusion, and diffusion through the film surrounding the particles, especially those regarding Eddy diffusion and intraparticle diffusion.

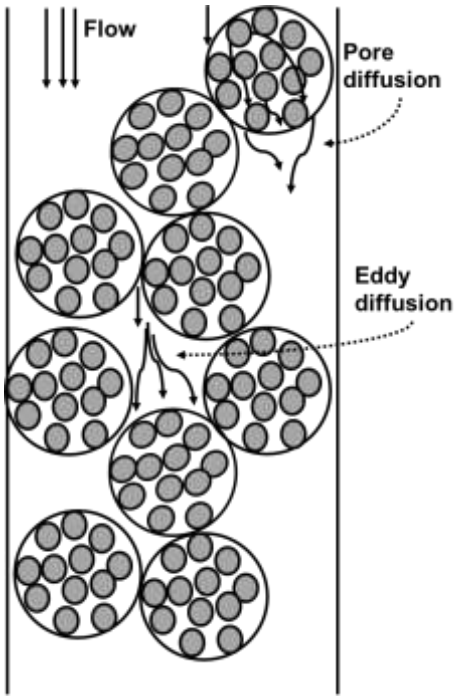



Figure 6.1: Scheme of Eddy and pore diffusion contributions to axial dispersion on a packed bed (adapted from Watler et al. (2002))

The derivation of the plate theory has been extensively described in the literature (Xu et al. 2013). The history and elution curve of a single band in partition chromatography are taken for demonstration, and $v_i$ and $v_{ii}$ are defined as the volumes of the moving and immobile phase in one theoretical plate. For a system with a distribution factor K, assumed to follow a linear profile of concentrations in both phases in the chromatographic separation, i.e., solid, and fluid, the effective plate volume, v, can be thus defined as:

$$v = v_i + \frac{v_{ii}}{K}, \text{ for which, } c_i = Kc_{ii}$$

For a system with feed concentration equals c0, and the feed volume equals A, the material balance derived for the first plate if a volume dS of the fluid phase passes through is as follows:

$$v_i dc_{i,1} + v_{ii} dc_{ii,1} + c_{i,1} dS = \begin{cases} c_0 dS \; for \; 0 \leq S \leq A \\ 0 \; for \; S > A \end{cases}$$

Similarly for the other plates, i.e., for n>1,

$$v_i dc_{i,n} + v_{ii} dc_{ii,n} + c_{i,n} dS = c_{i,n-1} dS$$

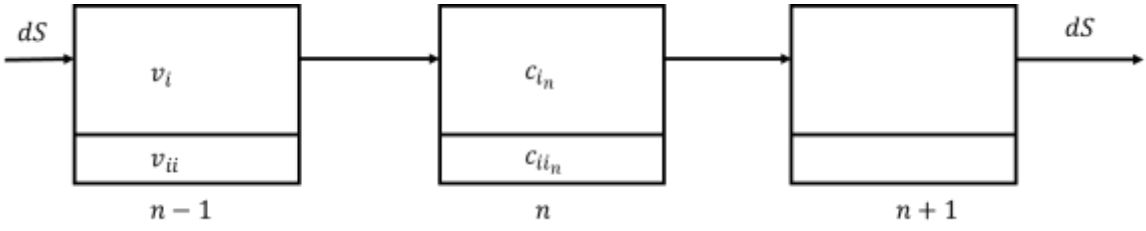



Figure 6.2.: Partition chromatography in stages (adapted from van Deemter et al. (1956))

The introduction of $y_n$ and w to the equations above, which simplify the concentration volumetric profile and the linear velocity, i.e., $y_n=c_{i,n}/c_0$, and w=S/v, the equations above are simplified to:

$$\frac{dy_1}{dw} + y_1 = \begin{cases} 1 \; for \; 0 \leq w \leq \dfrac{A}{v} \\ 0 \; for \; w > \dfrac{A}{v} \end{cases}$$

$$\frac{dy_n}{dw} + y_n = y_{n-1} \; (n > 1)$$

Initial conditions are $y_1=y_2=\ldots=y_n=0$ for w=0.

The solution for all stages can be written as:

$$y_n = \begin{cases} \int_0^w e^{-w'} \frac{w'^{n-1}}{(n-1)!} dw' for\ 0 \le w \le \frac{A}{v} \\ \int_{w-\frac{A}{v}}^w e^{-w'} \frac{w'^{n-1}}{(n-1)!} dw' for\ w > \frac{A}{v} \end{cases}$$

The integrand of $y_n$ for w>A/v is the Poisson distribution function, which can be approximated on conditions in which w is large enough, usually greater than 100, and qualitatively described if the number of plates is not too small (infinitely thin column) or if the band has arrived at the end of the column. Therefore, it can be assumed the following is true:

$$\frac{1}{n!} e^{-w'} w'^n \approx \frac{1}{\sqrt{2\pi w'}} e^{-\frac{(w'-n)^2}{2w'}}$$

For large values of w', it can be assumed that w'≈n, and that n-1≈n, and redefining w and yn as above, the van Deemter elution curve is described as:

$$c_{i,n} = \frac{c_0}{v\sqrt{2\pi n}} \int_{S-A}^{S} e^{-\frac{(\frac{S'}{v}-n)^2}{2n}} dS'$$

In order to relate width and height of an elution curve to the plate number, van Deemter defined the dimensionless quantities of s and a, as being:

$$s = \frac{S}{v\sqrt{n}}\ and\ a = \frac{A}{v\sqrt{n}}$$

Which rewrites the equation above as being:

$$\frac{c_{i,n}}{c_0} = \frac{1}{\sqrt{2\pi}} \int_{s-a}^{s} e^{-\frac{1}{2}(s'-\sqrt{n})^2} dS'$$

For the maximum value of $c_{i,n}/c_0$, the equation will be equivalent to an error function, i.e.:

$$\left(\frac{c_{i,n}}{c_0}\right)_{max} = erf\,\frac{a}{2\sqrt{2}}$$

Van Deemter also defines ∆S as being the width referent to the distance between the points of intersection of the tangents in the inflection points with the horizontal axis. For arithmetic purposes, a new implicit function δ is defined.

$$\delta e^{-\frac{\delta^2}{2}} = (a+\delta)e^{-\frac{1}{2}(a+\delta)^2}$$

Thus,

$$\Delta s = a + 2\delta + \sqrt{2\pi}\frac{a+\delta}{\delta}e^{\frac{\delta^2}{2}}\left(erf\,\frac{a+\delta}{\sqrt{2}} - erf\,\frac{\delta}{\sqrt{2}}\right)$$

Despite the mathematical complexity of ∆S, it can be easily seen that both height ($c_{i,n}/c_0$), and width (∆s) are only dependent on a. In summary, the efficiency of separation and recovery of a compound in chromatographic-based separations are only dependent upon the feed volume, the effective plate volume, and the number of theoretical plates.

If a continuous column was to be considered, i.e., the case in which a column does not fully saturate, a mass balance can be written on accordance to the visual representation on figure 6.2. Lapidus and Amundson (1952) described Eddy diffusion present in packed bed behavior, in which the longitudinal diffusion is caused by irregularities in the packing, which provide small signals to the overall diffusion coefficient.

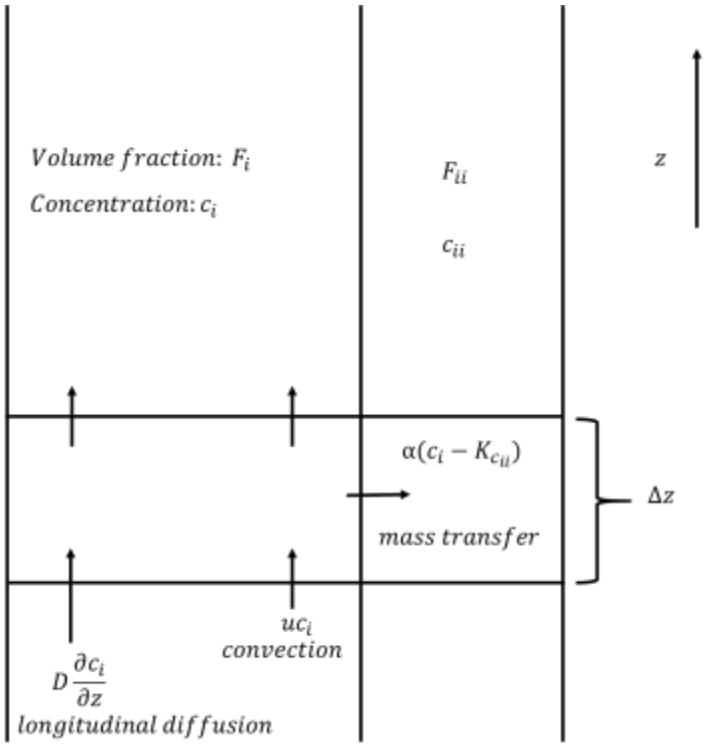



Figure 6.3: Material balance in a column (adapted from van Deemter et al. (1956))

For a cross sectional area on figure 6.2, it is seen that:

$$F_i \frac{\partial c_i}{\partial t} = F_i D \frac{\partial^2 c_i}{\partial z^2} - F_i u \frac{\partial c_i}{\partial z} + \alpha(K c_{ii} - c_i)$$

$$F_{ii} \frac{\partial c_{ii}}{\partial t} = \alpha(c_i - K c_{ii})$$

Lapidus and Amundson (1952) derived the solution for equation above, when a pulse of concentration $c_0$ during a short time $t_0$ was introduced to the system. Their solution is read as:

$$\frac{c_i}{c_0} = \frac{zt_0}{2t\sqrt{\pi Dt}} e^{-\frac{z(-ut)^2}{4Dt} - \frac{\alpha t}{F_i}}$$

$$+ \int_0^t \frac{zt_0 e^{-\frac{z(-ut')^2}{4Dt} - \frac{\alpha t}{F_i}}}{2t'\sqrt{\pi Dt'}} \sqrt{\left[\frac{\alpha^2 Kt'}{F_i F_{ii}(t-t')}\right]} e^{-\frac{\alpha K}{F_{ii}}(t-t') - \frac{\alpha t'}{F_i}} I_1 \left\{2\sqrt{\frac{\alpha^2 Kt'(t-t')}{F_i F_{ii}}}\right\} dt'$$

The general equation derived from Lapidus and Amundson (1952) can be simplified if a column has a large number of transfer units and mixing stages. The number of mixing stages can be defined for a column of height l as ul/2D, in which the height of a mixing stage being equal to 2D/u. The height of a transfer unit is defined by van Deemter as $F_i v/ \alpha$

### 6.4. Pressure drop considerations

Pressure drop is usually the limit that determines the size of a fixed bed chromatography column, usually being an important parameter to be considered when one is performing a resin screening. The Kozeny-Carman equation describes the pressure drop caused by the friction forces through an incompressible packed resin column:

$$\Delta P = u_0 L \frac{150\mu}{d_p^2}\left[\frac{(1-\varepsilon)^2}{\varepsilon^3}\right]$$

In which $u_0$ represents the superficial velocity of the mobile phase, L is the bed height, μ is the viscosity, $d_p$ is the particle diameter, and ε is the void fraction of the column (Watler et al. 2002).

The Kozeny-Carman equation has limited applications on ion-exchange medium, since most resin material are made from compressible polymeric material (reference). The matrix material in which resins are made of (e.g., polyacrylate)

will compress to an extent depending on the cross-linking, bed height, column diameter, and frictional properties of the medium. The Kozeny-Carman equation also does not account for the presence of a wall material, in which the resin beads are contained. Wall effects are clearly visible on narrow columns, and provide additional support for the column, thus, reducing bed compression. Different geometries will lead to different values of $u_{crit}$, which is the critical velocity, in which the pressure increases asymptotically, and no additional flow is achieved. Additional flow instability is found in the formation of small and highly compressed regions near the bottom of the column, which represent large deviations from the Kozeny-Carman equation. Colby et al. (1996) presented a model to account considering interstitial porosity, specific surface area, internal angle of friction, and the angle of wall friction. The three set of equations presented by Colby et al. (1996) are as follows, for frictional support, stress on the particles, and the pressure gradient, respectively:

$$\frac{\partial \tau_{zz}^{s}}{\partial z} = \mu u_0 \chi(\tau_{zz}^{s}) - \frac{4}{d_c} \tan \vartheta \frac{1 + \sin \xi}{1 - \sin \xi} \tau_{zz}^{s}$$

$$\chi(\tau_{zz}^{s}) = \frac{200 \varepsilon_s^2}{d_p^2 \phi^2 \varepsilon_f^3}$$

$$\frac{\partial P}{\partial z} = -\mu u_0 \, \chi(\tau_{zz}^{s})$$

In which $\tau_{zz}$ represents the stress acting on the resin particles in the axial direction, usually represented in Pa, z is the distance from the inlet of the column, $\chi(\tau_{zz})$ being an expression describing the contribution of porosity and specific surface area to the pressure gradient, dc is the column diameter, $\vartheta$ being the

angle of wall friction, ξ as the internal angle of friction, and ϕ as the shape factor of the particles ($m^{-1}$) (Colby et al. 1996).

The solution of these equations is often given by 4$^{th}$-order Runge-Kutta integration method, and several analyses have reported errors within the 20% range. Soriano et al. (1997) presented a column pressure drop model accounting gravity and the forces acting on the bottom, top, and the sides of a differential-sized slice of a column. The semi-empirical model accounts for the constant K from the Blake-Kozeny equation:

$$K = K_0 e^{-\left(\frac{P}{P_{s_0}}\right)}$$

And is written as:

$$\frac{dP_s}{dh} = \Delta\rho(1-\varepsilon)g + Ku_0 - P_s k \tan\vartheta \left(\frac{4}{d_c}\right)$$

In which, the new variables are K as the Blake-Kozeny constant, $K_0$ as the permeability of the uncompressed gel matrix ($m^2$), P as productivity (g $L^{-1}$ $h^{-1}$), $P_{s0}$ as the matrix rigidity (kPa), h as the reduced plate height (H/dp), Δρ as the density difference between the medium and the mobile phase, g as the acceleration due to gravity, k as a pressure coefficient, and $P_s$ as the verticle solid pressure in the bed (kPa).

The most appropriate way to solve the model derived by Soriano et al. (1997) is to employ 4$^{th}$-order Runge-Kutta integration methods, to generate axial pressure-flow curves for a given matrix and column specifications. Stickel and Fotopoulos (2001) introduced an empirical model to predict pressure drop from a series of

bed compression measurements – using different aspect ratios and column diameters. A linear model is, thus, presented and expects to linearly fit the critical velocities as a function of the aspect ratio; in which m and b are linear regression coefficients.

$$u_{crit} = \frac{m\left(\frac{L_0}{d_c}\right) + b}{L_0}$$

The critical bed compression $\varphi_c$ can be calculated as being:

$$\varphi_c = \frac{\varepsilon_c - \varepsilon_0}{\varepsilon_c - 1}$$

Which, for any given velocity lower than $u_{crit}$, the bed compression can be calculated as

$$\varphi = \varphi_c \frac{u}{u_c}$$

For which, m and b are linear coefficient parameters, and $L_0$ as the initial gravity-settled bed height.

Though pressure drop vs. flow curves are often obtained by the manufacturer, which can be used to estimate the maximum pressure a particular medium can hold, the operation conditions in columns may impose lower limits, especially because of wall effects. Typically, for large-scale fixed bed ion exchange systems, the operating flow rate will be determined according to the pressure drop limits, which allows retrofit calculations to determine the maximum column length for a given resin and backpressure limitations.

**6.5. Scale-up approach based on a volume basis**

The widespread use of van Deemter model on chromatographic scale-ups have helped much of the understanding in the application of industrial-sized chromatographic separations. Martin and Synge (1941) developed the concept of number of theoretical plates. While van Deemter's model describes the concept of height of a theoretical plate (H), Martin and Synge's approach described the relationship between the number of theoretical plates, N, to a column efficiency. N directly relates the column resolution, and while maintaining the column efficiency, a chromatographic system can be understood as a summation of individual plates. When adsorption-desorption-based chromatographic separations are considered, such as the case of ion exchange, for separation of multiple species that possess different chemical properties, e.g. pKa and ionic density, the separation performance will likely follow a linear scale up. This is true when it comes to maintaining similar condition of plates across a column, and possessing conditions to which the intraparticle diffusion rates are similar all across the column length.

The Péclet number (Pe) measures the macroscopic flow dispersion in a column, and is directly impacted by flow heterogeneity (Levenspiel and Levenspiel 1972). For a column system, Pe can be measured as:

$$Pe = \frac{uL}{D_a}$$

The Stanton number (St), on the other hand, describes the ratio of heat transferred into a fluid and its thermal capacity. For a resin system, it relates how

the flow influences the mass transfer within a column (Levenspiel and Levenspiel 1972).

$$St = \frac{6D_{pore}}{d_p Q}$$

Yamamoto et al. (1999) derived a relationship for Pe in the van Deemter equation, which, for a fixed Q, Pe ~ L/A. According to Hansen (2017), 1/CQ ~ St. The number of plates is an indication of separation in a packed-bed column, and the principle of scaling-up is based on the keeping N constant. Utilizing the discretion provided by the van Deemter model, N can be rewritten as:

$$N = \frac{1}{\frac{A}{L} + CQ}$$

In which Q=u/L is the column flow rate in BV per time unit. Hansen (2017) provides two major observations from the equation above: (i) for a fixed flow rate, Q, the plate number increases with the bed height, and (ii) if A/L << CQ, the plate number is independent of the bed height. Thus, if a fixed flow rate Q is selected, it is known that an increase in the bed height will, theoretically, provide similar or enhanced performance. This increase will follow an asymptotic profile, to which after a certain column length, the increase will be of low significance. If all operations are performed at a range greater than the minimum plate number, the performance should be satisfactory as long as Q is constant. Hansen (2017) describes such application to a few cases of protein separation. Therefore, the hypothesis from this demonstration presented is that, a scale up can be developed if St and Pe are maintained similar for the different scales.

## 6.6. Experimental

### 6.6.1. Column set up

Two chromatographic columns were used with diameters of 1 cm and 5 cm (ACE Glass, US). Thin stillage was obtained from a dry-grind ethanol plant in the state of Iowa, and was filtered to 0.45 µm using an ErtelAlsop 4T filter model. Filtered thin stillage was used as raw material for the experiments.

Columns were packed to different bed heights with Amberlite IRA 900 resin. Thin stillage was pumped at designed flow rates using a peristaltic pump. Nitrate and sulfate were analyzed with an Ion Chromatography system using a Dionex AS-11 column. Phytate was estimated using an adapted Wade method (Lorenz et al. 2007).

Isotherm experiments were performed utilizing different amounts of resin to a fixed amount of thin stillage. Breakthrough curves were plotted using sequential sampling from the column outlet. Breakthrough point was defined herein as the point in which the column efficiency is lower than 95%, i.e., $C/C_0>0.05$.

## 6.7. Results and discussion

### 6.7.1. Anionic competitiveness

The results presented on table 6.1 describe the maximum adsorption capacity of IRA 900 resin on thin stillage regarding nitrate, phosphate, and sulfate. The maximum adsorption capacity described herein is derived from the linearization of the multi-component Langmuir isotherm (data not shown). From this brief analysis, it can be seen that charge of the anion has some effect on the maximum adsorption capacity. For the species with lowest charge, nitrate, the

minimum capacity was found. Phytate, as described on chapter 5, has adsorption capacities greater than 120 mg $g^{-1}$.

Table 6.1. Langmuir-derived $q_m$ for nitrate, phosphate, and sulfate.

| Species | $q_m$ (mg $g^{-1}$) | $R^2$ |
|---|---|---|
| $NO_3^-$ | 36.28 | 0.9284 |
| $PO_4^{3-}$ | 68.98 | 0.9345 |
| $SO_4^{2-}$ | 47.35 | 0.9435 |

Marcus (1975) defined an equilibrium selectivity ratio, in which $z_A$ and $z_B$ are the charge numbers, [I±] represents the molar scale applied to the solution phase concentration. The mean molar coefficient, $y_{\pm IC}$ is also portrayed in the equation below:

$$k_{b,a} = \frac{\bar{x}_b^{\frac{1}{z_b}}[A_\pm]^{\frac{1}{z_a}} y_{\pm AC}}{\bar{x}_a^{\frac{1}{z_b}}[B_\pm]^{\frac{1}{z_b}} y_{\pm BC}}$$

Considering a constant $k_{b,a}$ as the equilibrium selectivity ratio per equivalent of ions A and B, and $x_B$ as the equivalent fraction of the B ion in the exchanger, a constant $k_0$ can be calculated as:

$$\log K^0 = \int_0^1 \log k_{b,a} d\bar{x}_b$$

$K_0$ represents, thus, a dimensionless equilibrium constant for the ion exchange reaction. -ΔH° represents the negative of the standard integral enthalpy. From a qualitative point of view, a summary of the anions present in thin stillage to which thermodynamic calculations were presented is reported on table 6.2. The anions selected herein are those found in thin stillage, to which thermodynamic data was reported in the literature. Though a thorough analysis cannot be done with these

results, it can be easily noted that increasing crosslinkage of the resin, an increase in exchange capacity of the ion B is increased.

Table 6.2: Literature data on $Cl^-$, $NO_3^-$, and $SO_4^{2-}$ ion exchange thermodynamic properties. Adapted from Aveston et al. (1958), Birch et al. (1959), Birch et al. (1967), Choppin et al. (1972), Gregor et al. (1955), Kühn et al (1969), Salmon (1969), Wheelwright and Roberts (1969), and Zalevskaya and Starobinets (1969).

| Ion A | Ion B | Crosslinkage (%) | Medium | Exchanger | log K ° | -ΔH ° (kcal) |
|---|---|---|---|---|---|---|
| $OH^-$ | $Cl^-$ | 8 | NR | $[-R-N(CH_3)_3^+-]_n$ | 0.38 | NR |
| $OH^-$ | $Cl^-$ | 2 | NR | $[-R-N(CH_3)_3^+-]_n$ | 1 | NR |
| $OH^-$ | $Cl^-$ | 4 | NR | $[-R-N(CH_3)_3^+-]_n$ | 1.26 | 1.8 |
| $OH^-$ | $Cl^-$ | 10 | NR | $[-R-N(CH_3)_3^+-]_n$ | 1.73 | NR |
| $Fl^-$ | $Cl^-$ | 10 | NR | $[-R-N(CH_3)_3^+-]_n$ | 0.9 | NR |
| $Fl^-$ | $Cl^-$ | | 0.1M $Na^+$ | $[-R-N(CH_3)_3^+-]_n$ | 0.57 | 1.55 |
| $Fl^-$ | $Cl^-$ | 1 | 0.1M $K^+$ | $[-R-N(CH_3)_3^+-]_n$ | 0.43 | 1.6 |
| $Cl^-$ | $NO_3^-$ | ~8 | NR | $[-R-N(CH_3)_3^+-]_n$ | 0.56 | 0.27 |
| $Cl^-$ | $NO_3^-$ 1/2 | 3 | NR | $[-R-N(CH_3)_3^+-]_n$ | 0.36 | NR |
| $Cl^-$ | $SO_4^{2-}$ 1/2 | | NR | $[-R-N(CH_3)_3^+-]_n$ | 0.06 | NR |
| $Cl^-$ | $SO_4^{2-}$ 1/2 | | NR | $[-R-N(CH_3)_3^+-]_n$ | 0.02 | NR |
| $Cl^-$ | $SO_4^{2-}$ 1/2 | | NR | $[-R-N(CH_3)_3^+-]_n$ | 0.02 | -1.1 |
| $Cl^-$ | $SO_4^{2-}$ | ~8 | NR | $[-R-N(CH_3)_3^+-]_n$ | 0.02 | -0.92 |
| $Fl^-$ | $Cl^-$ | 8 | 0.01-0.1M $Na^+$ or $K^+$ | $[-R-N(CH_3)_2(C_2H_4OH)^+-]_n$ | 1.09 | NR |
| $Cl^-$ | $NO_3^-$ | 8 | 0.01-0.1M $Na^+$ or $K^+$ | $[-R-N(CH_3)_2(C_2H_4OH)^+-]_n$ | 0.43 | NR |

| | | | | | | |
|---|---|---|---|---|---|---|
| $Br^-$ | $NO_3^-$ | 8 | 0.01-0.1M $Na^+$ or $K^+$ | NR | 0.02 | NR |

### 6.6.2. Scale-up analysis through breakthrough curves

The validation of the linear scale-up presented in this chapter is given as the measurement of the breakthrough curve under different experimental conditions.

Case 1: Effect of column height

Two runs were performed using a 1-cm packed column, one with column height of 2.5 cm (Q=2.75 BV $h^{-1}$) and one with column height of 5.0 cm (Q=1.35 BV $h^{-1}$), represented on figure 6.4 as white squares and black triangles, respectively. As it can be clearly seen, the breakthrough profile of both conditions is different. The 5.0 cm-column has a sharper profile, which indicates that its separation efficiency is increased. The 2.5 cm-column, in this run, performed with a wider band within the breakthrough zone. A number of factors, raised within the method development detailed in this chapter can explain the difference, and a summary is as follows: (i) the increase in Q will increase H within a column, thus, reducing its performance, (ii) an increase in the column height will increase N, and may reduce H, which increase the column performance.

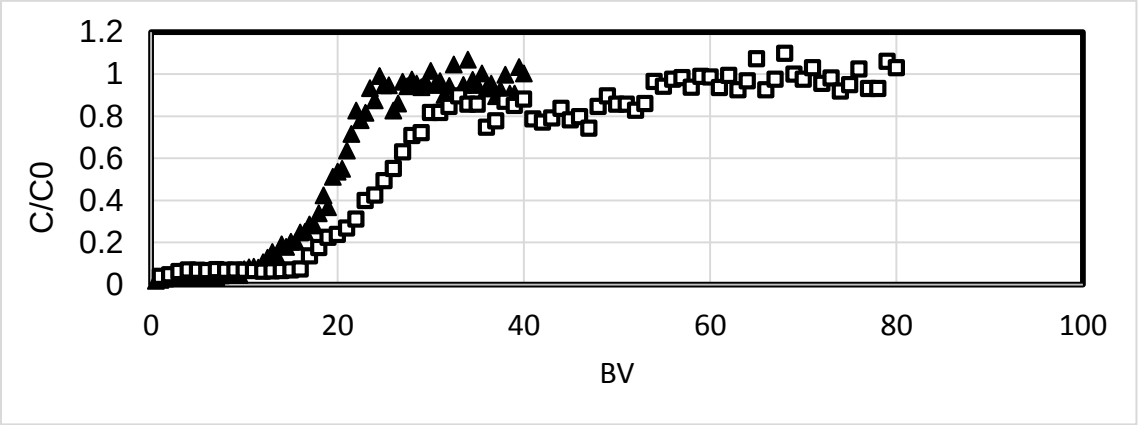



Figure 6.4: Effect of different column height on breakthrough curve

Case 2: Effect of similar Q for different heights

The results for case 2 are for the same Q (2.7 BV $h^{-1}$) on figure 6.5, but different column diameters and heights (5 cm height and 5 cm diameter for white diamond, and 2.5 cm height and 1 cm diameter for black circle). The results, as expected, show a similar breakthrough profile, since maintaining Q would, in accordance to Hansen (2017), maintain the plate number. The results clearly show that the operating condition is above the minimum Q and height, thus, indicating a linear scale-up approach based on the plate number.

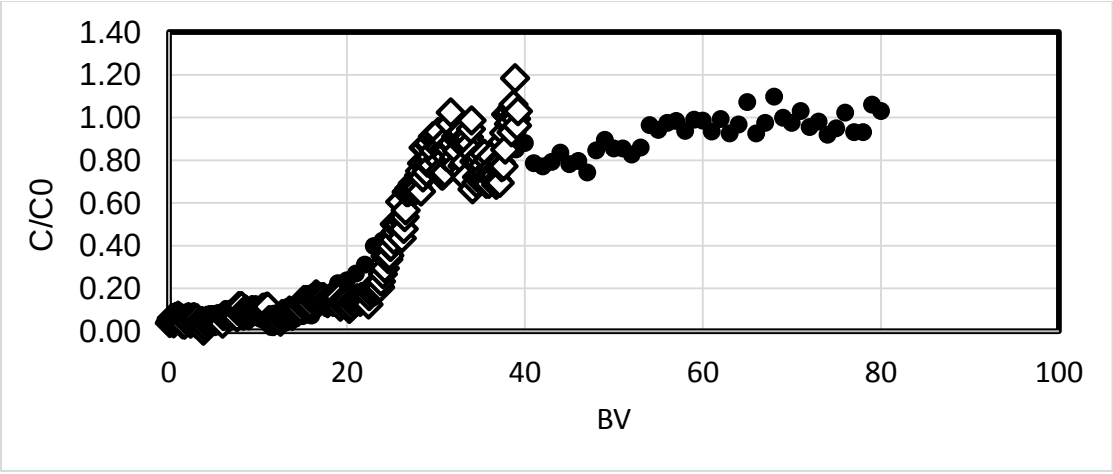


Figure 6.5: Effect of similar Q on scale-up for high-range Q.

Similarly, for a fixed Q (1.4 BV $h^{-1}$), and different column heights and diameters, a sharp and coincidental breakthrough profile is presented on figure 6.6. Similarly as figure 6.5, a fixed Q above the minimum requirement for linear scalability of plate numbers provides an easy and straight forward scale-up. However, it can be seen that a reduction in Q does have an impact on the plate height, which is indicated by a sharper breakthrough profile. Therefore, depending on the operational conditions, better performance of the column could be obtained with the conditions developed in this chapter, if Q is operated at lower levels.

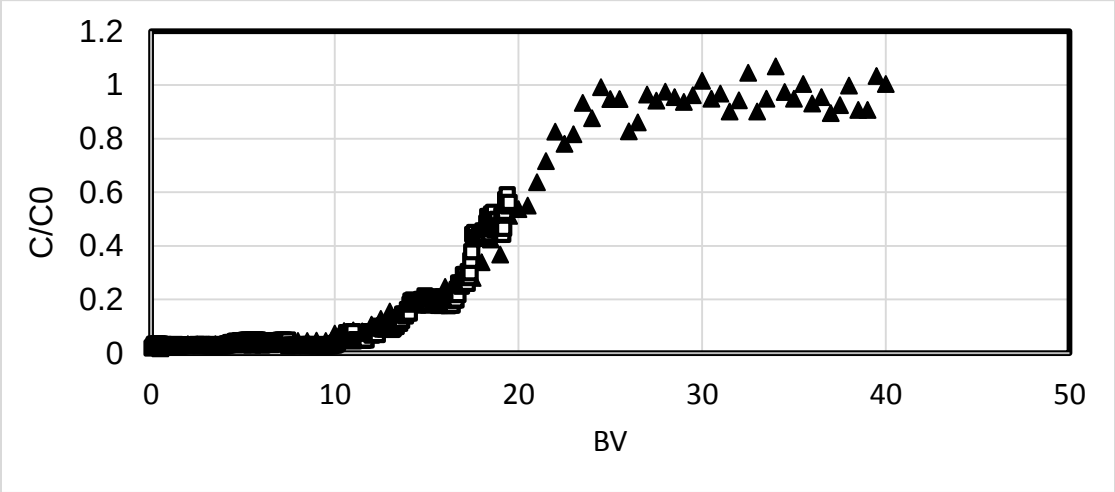



Figure 6.6: Effect of similar Q on scale-up for low-range Q.

**Conclusion**

Though it highly challenging to model a system with a wide variety of anions, including many of unknown composition (i.e. fragments of charged proteins, amino acids, hemicellulose and lignin fragments), a fundamental approach was taken to consider the scale-up of phytate extraction from thin stillage using IRA

900 resin. IRA 900 was proven in the previous chapters as an appropriate resin for phytate extraction, and showed significant selectivity towards phytate over other forms of phosphorus. The isotherm results presented herein indicate the selectivity of phytate over other anions (sulfate and nitrate) present in thin stillage. Based on previous studies on protein purification, a scale-up model based on Péclet number was derived for phytate extraction of thin stillage. The results show a similar breakthrough profile and similar breakthrough point range. Using extrapolation, further considerations can be made for possible pilot and industrial scale-up. Though it was not considered in this study, a summary of pressure drop considerations was also presented, since pressure drop is usually the limiting factor in scalability of ion exchange columns in an industrial scale.

# CHAPTER 7: ECONOMIC, ENVIRONMENTAL AND LIFE-CYCLE ASSESSMENT OF EXTRACTION OF HIGH-VALUE CHEMICALS FROM DRY-GRIND CORN-TO-ETHANOL PROCESS: A LOOK ON PHYTATE AND *MYO*-INOSITOL

## 7.1. Introduction

Corn-to-ethanol production has expanded on a fast pace in the United States, currently with production of over 15 billion gallons per year, accounting for over 50% of the world ethanol share(Reis et al. 2017). The well-established dry-grind process today accounts for also over 80% of the operating ethanol facilities in the United States, producing not only ethanol, but also distiller's grains. Distiller's grains, commonly commercialized as dried distiller's grains with solubles (DDGS) accounts for most of the heavy fraction from the ethanol distillation and rectification operations, and is processed through a complex downstream processing after the fermentation is unloaded(Belyea et al. 2004). Despite being a nutritious animal feed, inclusion of DDGS as feed ration is still limited due to

compositional properties, such as the high presence of non-starch polysaccharides, which are non-digestible to monogastric animals, the fatty acid profile, and the overdose of phosphorus, which is largely non-available to some animals(Spiehs et al. 2002). Phosphorus behavior in ethanol downstream processing is of particular interest, not only because it can lead to environmental issues, but also because it can potentially be an additional economic source to ethanol producers. Over 40% of the phosphorus present in ethanol coproducts is present as phytate, the main form of organic phosphorus in cereals and grains, which is not completely hydrolyzed during the whole ethanol process(Alkan-Ozkaynak and Karthikeyan 2012; He et al. 2017b; Reis et al. 2017; Spiehs et al. 2002). Phytate has a high market value, and today is a platform chemical utilized in a wide array of industries, and it is mostly exploited because of its high chelating potential(He et al. 2017a). Phytate is also the raw material to produce *myo*-inositol, a cyclic polyol with a variety of uses by pharmaceutical and nutraceutical companies(Maga 1982).

Phytate is found to be dissolved on the ethanol coproducts(He et al. 2017a). During the dry-grind downstream processing, thin stillage, i.e., the liquid-rich fraction derived from the centrifugation of whole stillage, accounts for the highest concentration of phytate among all the coproducts. Recent research (He et al. 2017a; Hu and He 2016) has proposed an extraction method using ion exchange separation, which can efficiently separate phytate from thin stillage, with little to no effect on the residual nutritional profile of the final DDGS. Even though the

concentration of phytate being relatively low on ethanol coproducts, at around 1 g $L^{-1}$ in thin stillage, due to the enormous volumetric production by dry-grind plants, the production leveraging costs in extracting phytate might become an industrial alternative.

The high volumes of DDGS produced by ethanol plants, alongside with its limited dietary inclusion, pose a challenge for higher revenues to ethanol plants. The second-most important co-product from dry-grind ethanol facilities, distiller's corn oil, represent today a mature product suitable for biodiesel production and other uses of low-grade vegetable oil(Reis et al. 2017). Removal of distiller's corn oil, on the other hand, has been criticized by animal nutritionists claiming a loss in energy value from DDGS. Despite still representing about 20% of the total revenue stream from dry-grind ethanol plants, DDGS are still ranking in low positions when compared to higher end animal feeds, such as soybean meal(Lupitskyy et al. 2015). The extraction of high-value chemicals through mature sound technologies may represent, thus, an alternative for higher economic security of ethanol plants. This current article describes a techno-economic analysis and a life-cycle assessment of a phytate extraction process on a large-scale dry-grind corn-to-ethanol plant in the United States, and its posterior *in-loco* conversion to *myo*-inositol. Since the hydrolysis of phytate to *myo*-inositol has been previously explored (Phillipy et al. 1987) empirical data was collected to test the precise purities and yields after phytate was produced.

## 7.2. Materials and methods

### 7.2.1. Process development

The scope of this work analyzed the utilization of corn-to-ethanol thin stillage produced through the dry-grind process as raw material to extract phytate using an ion exchange process, followed by hydrolysis to generate *myo*-inositol (He et al. 2017). The process was modelled according to He et al. (2017) and Hu and He (2016), and by additional empirical analysis, when required. Thin stillage was obtained from a dry-grind ethanol plant in the state of Iowa, USA. The process of converting thin stillage to a phytate brine solution is divided into the major steps: (1) filtration, (2) flow through ion exchange column, (3) desorption of phytate using brine solution, (4) reconditioning of column using sodium hydroxide, and (5) column washing using water. The conversion of phytate brine solution to calcium phytate is done by (6) addition of calcium chloride and sodium hydroxide, followed by product filtration, and production of *myo*-inositol is achieved by (7) hydrolysis of phytate brine solution, (8) precipitation of phosphate with calcium chloride and sodium hydroxide, and (9) evaporation and drying of *myo*-inositol.

(1) Filtration. Thin stillage is characterized a liquid-rich material, with solid content values ranging from 5 to 7%. The presence of solids is highly undesirable to a prolonged lifespan of the ion exchange resin, since they can build up during the percolation on the column pathway and increase fouling rates. Therefore, micron-filtration is required to remove the solids. Data from Arora et al. (2010) was used in the filtration estimates, and it was assumed that solids could be redirected to the unmodified dry-grind process, thus, not affecting the production of distiller's grains. It was also

assumed that the volume of thin stillage prior and after filtration did not alter significantly, thus, the inlet liquid volumetric flow was the same as the outlet liquid flow.

(2) Ion exchange process. Amberlite IRA 900 has been described by Hu and He (2016) as an optimum ion exchange resin for phytate extraction from stillage water. Industrial-sized columns were estimated to have similar capacity as those tested by Blaney et al. (2007) , and that the reconditioning of resin would promote a long lifespan. Thin stillage would flow through a packed-bed column with Amberlite IRA 900 resin. Saturation was estimated at conditioning of 13 bed volumes, and it was assumed that 95% of phytate phosphorus from the processed thin stillage would be adsorbed onto the resin beads (Hu and He 2016). Temperature and pH adjustment has not been considered in the assumptions, since Amberlite IRA 900 resin has robustness to hold temperatures up to 70 ºC, close to the processing temperature of thin stillage on a traditional dry-grind ethanol plant(Reis et al. 2017). Thin stillage liquid was estimated to return to traditional dry-grind downstream processing, thus, not affecting the overall system.

(3) Desorption of phytate. Once the resin is saturated, it can be desorbed using a sodium chloride solution at a concentration of 2 mol $L^{-1}$ (Hu and He 2016). Hu and He (2016) also described the volume of NaCl solution to be used within the conditions tested necessary to desorb 100% of the

phytate bound to the ion exchange resin beads, producing a phytate solution at concentration of 12 g $L^{-1}$.

(4) Reconditioning of resin. Once the resin is spent, and phytate has been desorbed, it must go through regeneration with NaOH, as described by the manufacturer's manual. The high ionic strength of NaOH in this process regenerates the spent exchange sites, recovering them to an exchangeable state (Marais and Nyokong 2008).

(5) Column washing. Contact of residual sodium hydroxide with fresh thin stillage can promote changes in the nutritional profile of thin stillage (Felix et al. 2012). Leftover sodium hydroxide can also promote the change of pH in thin stillage, likely affecting the ion exchange process, due to the wide range of pKa of phytate (Cigala et al. 2010; Evans and Pierce 1981). Therefore, a reconditioning with water was considered necessary in order to maintain feedstock homogeneity to posterior downstream processing of spent thin stillage.

(6) Precipitation of phytate. Phytate can be precipitated as a calcium salt in pH ranges close to neutral values (He et al. 2017). He et al. (2017) precipitated phytate from a NaCl solution using calcium chloride addition and promoting a pH shift using NaOH. It is assumed that a ratio of 1 mol/mol of $Ca^{2+}$ being required to recover phytate phosphorus (He et al. 2017). Calcium phytate is a stable solid material under neutral pH conditions, and can be recovered via filtration, and gentle drying.

(7) Hydrolysis of phytate to generate *myo*-inositol and phytate. The hydrolysis of phytate to generate *myo*-inositol has been exploited via acid hydrolysis. However, no literature data is available regarding the conditioning and behavior of phytate in presence of NaCl. Experiments were carried out to test appropriate conditions, such as time of reaction, pH, and temperature on the hydrolysis, and empirical results were used on process modeling.

(8) Recovery of phosphate from phytate hydrolysis. Once hydrolysis to generate *myo*-inositol is complete, a ternary system with *myo*-inositol, phosphate, and NaCl is generated. As all of these compounds are soluble at the reaction conditions, recovery of phosphate has been estimated to occur on a similar process as described in (6), i.e., via addition of calcium cations, and precipitation of calcium phosphate salts under neutral pH. Calcium phosphate salts are insoluble under neutral pH and can be recovered on a similar process as also described in (6), via filtration and gentle drying.

(9) Recovery of *myo*-inositol. Myo-inositol is soluble in water (Holub 1986), and recovery is proposed via desalination, evaporation, and drying. Desalination is required to remove NaCl from solution, thus providing *myo*-inositol in water.

### 7.2.2. Economic considerations

A techno-economic model developed by researchers at the department of Applied Economics at the University of Minnesota, BuGal, was used as basis for modification (Tiffany and Eidman 2003). The BuGal model was developed with

industrial, brokers, and academic collaborations and has been extensively used by economic considerations in the dry-grind industry(Cardona and Sánchez 2007; Rajagopal and Zilberman 2008; Sanchez and Cardona 2008). The economic model considers the production of denatured gallons of ethanol as product outcome, and bushel of corn ground as main feedstock income. BuGal model considers five measures of cost, and include on a per unit basis: gross margin, total energy cost, total chemical cost, total processing cost, and net margin achieved. The data inputs can be source of input on a timely manner (e.g. monthly or yearly), and are linked to the net margin achieved on a bushel basis. BuGal can control the gross margin per bushel, i.e., relating the summed value of products sold by a plant (ethanol, DDGS, $CO_2$, and modified in the case for this article, phytate, and *myo*-inositol) subtracted from the cost of key feedstock. The overall operating costs are divided into several categories: energy, chemicals, depreciation, maintenance, labor, interest, and additional expenses, such as depreciation of plant and equipment, repairs, quality control costs, insurance, licenses, and real estate taxes. Two major categories of energy sources are considered in the model and are natural gas and electricity. The current study only considers the basis of comparison among different scenarios within the same plant, thus, not modifying the expenses under the category of additional expenses.

A model plant with ethanol production capacity of 100 million gallons per year was considered in the model. Initial assumptions on deriving the installed costs

and details on the major assumptions for operating and capital costs is found on table 7.1.

Table 7.1.: Assumptions used in BuGal method – Adapted from Tiffany and Eidman (2003).

| | |
|---|---|
| Debt-Equity Assumptions | |
| Factor of Equity (2007 Plants Only) | 70% |
| Factor of Debt (2007 Plants Only) | 30% |
| Interest Rate Charged on Debt | 7% |
| Investor Required Return on Equity | 12% |
| SL Depreciation based on asset life (years) | 15 |
| | |
| Key Assumptions Applied | |
| Installed Cost: Phytate Extraction | $2,750,000 |
| Percent of Thin Stillage Processed | Variable |
| Installed Cost -Inositol & Fertilizer Proc. | $930,000 |
| % of Calcium Phytate to Inositol | Variable |
| Output Market Prices | |
| Ethanol Price (denatured price) $/gal. | $1.70 |
| DDGS Price $/T | $161.00 |
| Price Factor Reduced Phytate DDGS | 1.00 |
| Reduced Phytate Feed Price | $161.00 |
| Calcium Phytate Price ($/ kilogram) | $ 12.00 |
| CO2 Price ($ per Ton liq. CO2) | $3.00 |
| Corn Oil Extracted Price at Plant $/lb | $0.165 |
| Inositol Powder Sold ($/ kg.) | $10.00 |
| Calcium Phosphate Ferilizer Sold $/ U.S. Ton | $200.000 |
| Soybean Oil Price per Pound | $0.33 |
| Feedstock Delivered Price Paid by Processor | |
| Corn Price ($ per bu.) | $4.00 |
| Corn Oil Percentage in Corn Delivered | 3.00% |
| Corn Oil % Extracted by Centrifuge | 28% |
| Energy Prices | |
| Natural Gas Price ($ per 1,000,000 Btu) | $6.00 |

| | |
|---|---|
| Electricity Price (Plant is Buyer) ($ per kWh) | $0.07 |
| LP (Propane) Price ($ per gallon) | $2.00 |
| Factor of Elect. Energy Reqd-Inositol&Fert Prod | 1.025 |
| Factor of Thermal Energy Reqd Inositol + Fert | 1.000 |
| | $ |
| Calcium Chloride ($/Ton) | 136.00 |
| Denaturant Price / gal | $2.50 |
| Denat/100 gal Anhyd. | 2 |
| Factor of Labor Requirement--Oil Extraction | 1.05 |
| Factor of Labor Reqd. - Oil +Phytate Extraction | 1.10 |
| Factor of Labor Reqd.-Oil +Inositol +Fertilizer | 1.00 |
| Feedstock-to-Ethanol Conversion Yields | |
| Ethanol Yield--Corn (anhydr. gal per bu) | 2.795 |
| DDGS lb. per bushel of corn | 17.5 |
| CO2 lb. per bushel of corn | 17.5 |

### 7.2.3. Costs of installed phytate extraction unit and conversion to *myo*-inositol

The assumptions used to build the capital and operating costs of the proposed extraction method of phytate and posterior conversion to *myo*-inositol are based on (Strathmann 2004; Wachinski 2016). Conservative values of extra 2.5% additional energy to the total plant being utilized to power the additional processing were used. The proposed system accounts for a separation of thin stillage to the filtration system, in order to remove particles that can cause negative effects on the ion exchange extraction columns. Amounts of thin stillage from 5-20% from the total flow were used in the estimation. The capital cost for installing a phytate extraction unit considered a maximum flow of 20% of the total thin stillage produced by a 100-million per year ethanol plant, and it was

estimated that production of calcium phytate or *myo*-inositol could be interchanged at any ratio, i.e., the proposed system could either produce 100% of calcium phytate or 100% of *myo*-inositol, or any ratio in between.

### 7.2.4. Life cycle assessment on phytate extraction

The life cycle assessment of calcium phytate extracted from corn-to-ethanol thin stillage was estimated using GREET model. Calcium phytate extraction, and posterior conversion to *myo*-inositol were implemented on two stationary models on GREET – Dry mill ethanol production w/ corn oil extraction(Arora et al. 2008; Lampert et al. 2015; Shapouri et al. 2002; Wang et al. 2012; Wang et al. 2011; Wang et al. 1999; Wang et al. 2014), and Dry mill ethanol production w/o corn oil extraction(Arora et al. 2008; Lampert et al. 2015; Shapouri et al. 2002; Wang et al. 2012; Wang et al. 2011). Since the basis of the BuGal model for techno-economic analysis has been modified to analyze both conditions (with and without distiller's corn oil extraction), the two stationary models on GREET to describe corn-to-ethanol process were utilized.

### 7.2.5. System boundaries, life cycle inventory, and functional unit

As a matter of consistency to the BuGal techno-economic model, it evaluates the steps as corn enters the factory to the point that denatured ethanol is produced. The functional unit considered, also for a matter of consistency with the BuGal model, is a gallon of denaturated ethanol produced by a corn-to-ethanol dry-grind plant. The life cycle inventory for this study was based on estimates from GREET database. Calcium phytate was considered as a displaced co-product in the modified GREET model.

### 7.2.6. Conversion of phytate to *myo*-inositol

Phytate present as eluent from the ion exchange process was used as feedstock to further conversion to *myo*-inositol. Acid hydrolysis was performed on screw-top tubes with 3 mL of reaction volume. The reaction pH, when needed, was adjusted with HCl 3 mol $L^{-1}$. A full factorial design and a central composite design were using to evaluate pH, temperature, and time.

HPLC measurement of *myo*-inositol was performed using an Agilent HPLC 1200 module loaded with an Aminex® HPX-87H column (300 x 8.7 mm), in series, with a Bio-Rad Micro-Guard Cation H Refill Cartridges (30 x 4.6 mm) (Bio- Rad Laboratories, Hercules, CA) guard column (Noureddini and Dang 2009). The column temperature was maintained at 65 °C, and a refractive index detector, held at 30 °C. The mobile phase was a 0.5 mmol $L^{-1}$ sulfuric acid solution. Analysis was performed isocratically by delivering the mobile phase at a flow rate of 0.4 mL/min. Total run time for this method was 20 min. The elution time for *myo*-inositol was about 9 min. The injection volume of standards and sample solutions were 20 µl.

## 7.3. Results and discussion

### 7.3.1. Economic analysis

Unlike major recent upgrades to the dry-grind ethanol industry, such as distiller's corn oil removal, extraction of phytate and production of *myo*-inositol are related to products found in dilute concentrations. The low concentration of phytate in ethanol coproducts requires low energy input on an ethanol basis, while still providing a high-value co-product. BuGal model calculates the operating expenses per gallon of denatured ethanol sold as USD 0.2673 for the control

conditions, i.e., without phytate extraction, and USD 0.2686 for a system considering 20% of thin stillage being redirected to phytate extraction, due to increase in energy consumption for additional processing. Regarding chemical costs, phytate extraction and conversion to inositol likely increased the value from USD 0.1201 to USD 0.1226 per gallon of denatured ethanol, mainly due to the requirement of NaCl for phytate desorption from the ion exchange process, additional process water, and calcium chloride addition. These costs are balanced by the calcium phytate, *myo*-inositol, and calcium phosphate as fertilizer (derived from the phytate hydrolysis) sales, at prices estimated at USD 12, USD 10, and USD 0.20 per kg, respectively. BuGal estimates an average large-scale dry-grind operation, with factor of equity and debt being 0.7 and 0.3, respectively, with an interest rate charged on debt as 0.07, and the rate of return required by investors on the plant's equity as 0.12. Even though these numbers are variable, the economic considerations on such assumptions are beyond the scope of this work, and remain constant through all assumptions. Following similar numbers as Kwiatkowski et al. (2006), 2.795 gallons of ethanol are estimated to be produced from each bushel of corn, and 17.5 lb of DDGS, and $CO_2$ are produced on a bushel basis. Denatured ethanol sales are estimated to be at USD 1.70 per gallon, an estimate within current market prices , and that phytate extraction would not affect the price of DDGS, thus, being marketed at a price of USD 161 per ton, also within current market estimates (Popp et al. 2016).

Table 7.2. BuGal results for simulation for 20% of thin stillage diverted to phytate extraction unit, and 0% of the extracted phytate being redirected to myo-inositol production.

| | Cost/Denat. Gal. Ethanol | | | 2/2/2017 | Plant Totals |
|---|---|---|---|---|---|
| Nameplate Ethanol Production | 100,000,000 | | $ 124,000,000 | | |
| Investment per Nameplate Gallon | $1.277 | | $3,680,000 | Plant Cost | $ 127,680,000 |
| Factor of Nameplate Capacity | 1.000 | 20% | % of Thin Stillage Processed to Phytate | | |
| Debt-Equity Assumptions | | | | Plant Profits | $ 23,535,390 |
| Factor of Equity | 0.70 | 0% | % of Phytic Acid to Inositol + Fert | | |
| Factor of Debt | 0.30 | | | Initial Debt | $ 38,304,000 |
| Interest Rate Charged on Debt | 0.07 | | | Payback of Addl Invest. | 1.01 |
| Rate of Return Reqd. by Investors on Equity | 0.12 | | | Rate of Return | 18.43% |
| Conversion Efficiency Assumptions | | | Annual Production | | |
| Anhydrous Ethanol Extracted (Gal. per Bu.) | 2.795 | | Bushels Ground | Denat. Gallons | Tons of LowPHY DDGS Sold |
| Low Phytate DDGS | 17.5 | | 35,090,133 | 100,000,000 | 299,557 |

| | | | | | |
|---|---|---|---|---|---|
| CO2 extracted per Bushel (lb. per Bu.) | 17.5 | | CO2 Sold(Tons) | Calcium Phytate (kg) | Crude Corn Oil (lb.) |
| Corn Oil Percent of Corn D.M | 3.00% | 0.28 | 307,039 | 469,127 | 14,195,503 |
| Factor of Phytate Converted to Inositol | 50.00% | | | Inositol (kilograms) | - |
| | | | | Cal Phosphate Fert (kg) | - |
| Establishment of Gross Margin | Price per Unit | | Tons Revenue/Bu. Ground | Revenue/Gal. Denatured Sold | Plant Totals |
| Ethanol Price (denatured price) $/gal. | $1.70 | | $4.8447 | $1.7000 | $ 170,000,000 |
| Low Phytate Distillers price per ton | $161.00 | 100% | $1.3744 | $0.4823 | $ 48,228,632 |
| Corn Oil Sales ($/lb) | $0.165 | | $0.0667 | $0.0234 | $ 2,342,258 |
| Calcium Phytate Sales per Kilogram | $ 12.00 | | $ 0.1604 | $ 0.0563 | $ 5,629,524 |
| Inositol Sales, Price per Kilogram | $ 10.00 | | $ - | $ - | $ - |
| Calcium Phosphate Fertilizer per Ton | $ 0.20 | | $ - | $ - | $ - |
| CO2 Price ($ per Ton liq. CO2) | $3.00 | | $0.0263 | $0.0092 | $ 921,116 |
| Federal Small Producer Credit | $0.00 | | $0.0000 | $0.0000 | $ - |
| Sales of Solubles ($/ Ton) | $ - | | $0.0000 | $0.0000 | $ - |

| | | | | | |
|---|---|---|---|---|---|
| Max.Prem for Low-Carbon Imprint for Ethanol | $0.00 | 0 | $0.0000 | $0.0000 | $ - |
| Revenue per Unit | | | $6.4725 | $2.2712 | $ 227,121,529 |
| Corn Price Paid by Processor ($ per bu.) | $4.00 | | $4.0000 | $1.4036 | $ 140,360,533.92 |
| Gross Margin | | | $2.4725 | $0.8676 | $ 86,760,995.21 |

| Operating Expenses Per Bushel | Price per Unit | Cost /Bushel Ground | Cost /Gal. Denatured Sold | Plant Totals |
|---|---|---|---|---|
| Natural Gas Price ($ 1,000,000 Btu) | $6.00 | $0.5697 | $0.1999 | $ 19,992,000 |
| LP (Propane) Price ($ per gallon) | $2.00 | $0.0423 | $0.0024 | $ 1,484,716 |
| Factor of Time Operating on Propane | 0.02 | | | |
| BTU's of Heat fr Fuel Req./ Denat. Gal. | 34,000 | | | |
| Combined Heating Cost | | $0.6120 | $0.2148 | $ 21,476,716 |
| Electricity Price ($ per kWh) | $0.07 | | | |
| Kilowatt Hours Required per Denat.Gal. | 0.769 | | | |
| Electrical Cost | | $0.1534 | $0.0538 | $ 5,381,250 |
| Total BTU's of Fuel and Electricity | 41,688 | | | |

| | | | | | |
|---|---|---|---|---|---|
| Total Energy Cost | | | $0.7654 | $0.2686 | $ 26,857,966 |
| | Cost/Denat. Gal. Ethanol | | | | |
| Enzymes | $0.0400 | | $0.1140 | $0.0400 | $ 4,000,000 |
| Yeasts | $0.0040 | | $0.0114 | $0.0040 | $ 400,000 |
| Salt for Elution | | | $0.0000 | $0.0000 | $ - |
| Other Proc.Chemicals & Antibiotics | $0.0200 | | $0.0570 | $0.0200 | $ 2,000,000 |
| Boiler & Cooling Tower Chemicals | $0.0050 | | $0.0142 | $0.0050 | $ 500,000 |
| Water | $0.0030 | | $0.0085 | $0.0030 | $ 300,000 |
| Denaturant Price per Gal. | $2.50 | 2 | $0.1370 | $0.0481 | $ 4,807,692 |
| Calcium Chloride Purchase | $ 136.00 | /tonne | $0.0000 | $0.0000 | $ - |
| Total Chemical Cost | | T. | $0.3422 | $0.1201 | $ 12,007,692 |
| Depreciation based on C49 asset life | 15 | | $0.2426 | $0.085120 0 | $ 8,512,000 |
| Maintenance & Repairs | $0.0400 | | $0.1140 | $0.0400 | $ 4,000,000 |
| Interest Expense | | | $0.0764 | $0.0268 | $ 2,681,280 |

| | | | | |
|---|---|---|---|---|
| Labor | $0.0550 | $0.1567 | $0.0550 | $ 5,500,000 |
| Management & Quality Control | $0.0167 | $0.0475 | $0.0167 | $ 1,666,667 |
| Real Estate Taxes | $0.0020 | $0.0057 | $0.0020 | $ 200,000 |
| Licenses, Fees& Insurance, Waste Mgmt. | $0.0140 | $0.0399 | $0.0140 | $ 1,400,000 |
| Other Expenses | $0.0040 | $0.0114 | $0.0040 | $ 400,000 |
| Total of Other Processing Costs | | $0.6942 | $0.2436 | $ 24,359,947 |
| Total Processing Costs | | $1.8018 | $0.6323 | $ 63,225,605 |
| Net Margin Achieved Per Unit | | $0.6707 | $0.2354 | $ 23,535,390 |
| Investor Reqd. Return on Equity | 12.00% | $0.3056 | $0.1073 | $ 10,725,120 |
| Increment of Success/Failure to Meet Required Return | | $0.3651 | $0.1281 | $12,810,270 |

Figure 7.1 analyzes the insertion of values ranging from 5 to 20% of thin stillage being processed to phytate extraction, and values from 0 to 100% of the extracted phytate converted to *myo*-inositol. The response observed on figure 7.1 is a comparison with the internal rate of return observed from a plant with and one without corn oil extraction, in which the light gray indicate ranges with rate of return greater than the control, while those in dark grey indicate rates of return lower than the control. As the price of *myo*-inositol is lower than that of phytate, any increase in the production of *myo*-inositol will provide lower returns when compared to the phytate control conditions.

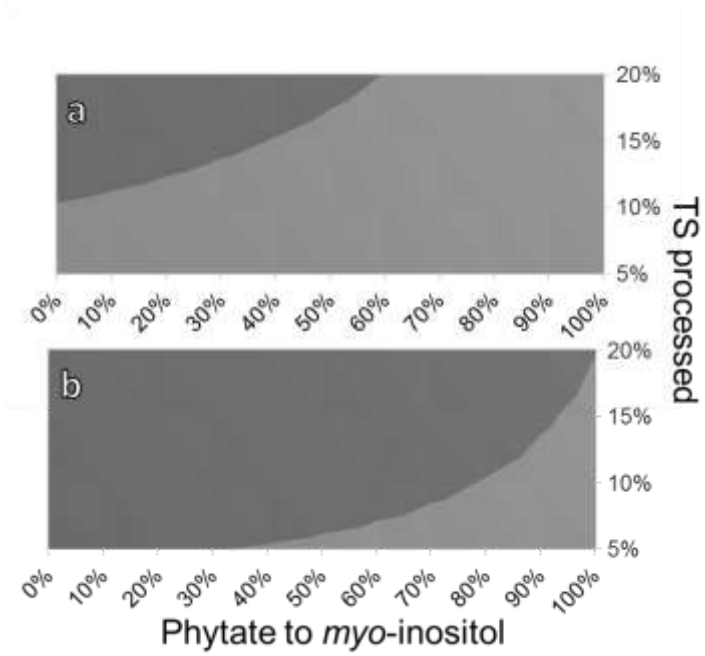



Figure 7.1.: sensitivity analysis using different amounts of TS processed through ion exchange process and different amounts of the extracted phytate being converted to myo-inositol for a plant (a) with distiller's corn oil extraction, and (b) without distiller's corn oil extraction

### 7.3.2. Phytate conversion to *myo*-inositol

Acid hydrolysis was chosen as method of production of *myo*-inositol from phytate due to high kinetic rates, low cost of production, and ease of operation in batch and industrial scales. The results from the full factorial design infer that the hydrolysis, under the conditions tested, is preferred at pH = 3.7±0.2. This results is in consonance to the hydrolysis performed by Phillipy et al. (1987) on sodium phytate. The second full factorial results indicate that, at pH 3.7, temperature should be leveled at 150 ºC and time at 45 min.

Table 7.3 Full factorial analysis on pH and time effect on phytate hydrolysis

| Experiment | Time (min) | pH | Yield (%) | Experiment | Time (min) | pH | Yield (%) |
|---|---|---|---|---|---|---|---|
| 1 | 5 | 1 | 9 | 17 | 5 | 1 | 22.6 |
| 2 | 5 | 2.5 | 9.1 | 18 | 5 | 2.5 | 8.9 |
| 3 | 5 | 3.7 | 10.1 | 19 | 5 | 3.7 | 9.8 |
| 4 | 5 | 5.5 | 9.7 | 20 | 5 | 5.5 | 9.6 |
| 5 | 15 | 1 | 16.3 | 21 | 15 | 1 | 15.9 |
| 6 | 15 | 2.5 | 22.8 | 22 | 15 | 2.5 | 20.1 |
| 7 | 15 | 3.7 | 27.6 | 23 | 15 | 3.7 | 25.6 |
| 8 | 15 | 5.5 | 23.5 | 24 | 15 | 5.5 | 22.4 |
| 9 | 25 | 1 | 31.3 | 25 | 25 | 1 | 32.1 |
| 10 | 25 | 2.5 | 41.4 | 26 | 25 | 2.5 | 45.7 |
| 11 | 25 | 3.7 | 58.1 | 27 | 25 | 3.7 | 51 |
| 12 | 25 | 5.5 | 36.3 | 28 | 25 | 5.5 | 33.1 |
| 13 | 45 | 1 | 76.4 | 29 | 45 | 1 | 83.2 |
| 14 | 45 | 2.5 | 98.9 | 30 | 45 | 2.5 | 95 |
| 15 | 45 | 3.7 | 94.9 | 31 | 45 | 3.7 | 98.6 |
| 16 | 45 | 5.5 | 76.1 | 32 | 45 | 5.5 | 62.4 |

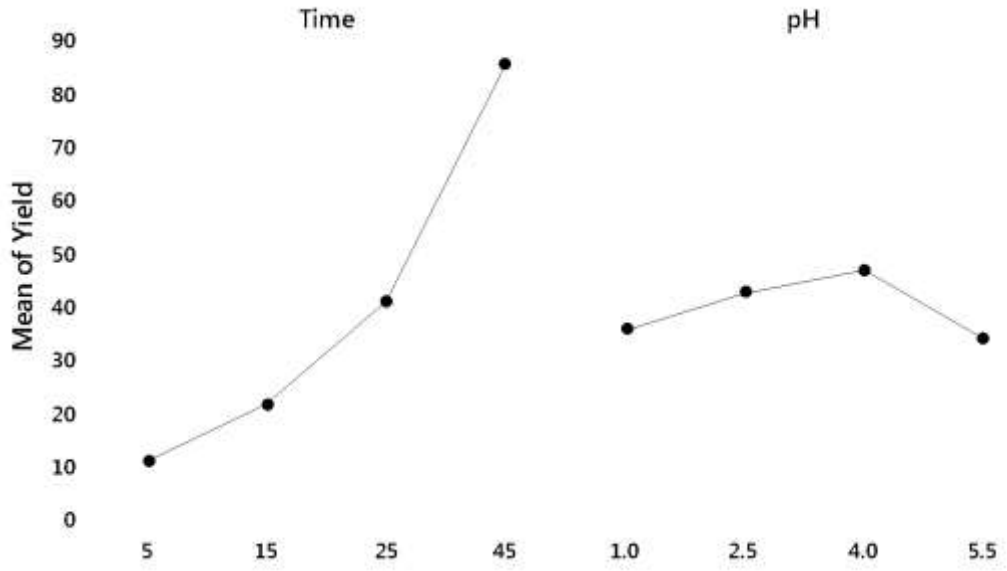



Figure 7.2: Plot of main effect plots of time and pH towards yield (fixed time)

Table 7.4 Full factorial analysis on temperature and time effect on phytate hydrolysis for fixed pH = 3.7

| Experiment | Temperature (ºC) | Time (min) | Yield (%) | Experiment | Temperature (ºC) | Time (min) | Yield (%) |
|---|---|---|---|---|---|---|---|
| 1 | 60 | 5 | 0.3 | 13 | 60 | 5 | 1.2 |
| 2 | 60 | 15 | 14.6 | 14 | 60 | 15 | 14.4 |
| 3 | 60 | 25 | 14.6 | 15 | 60 | 25 | 14.6 |
| 4 | 60 | 45 | 14.6 | 16 | 60 | 45 | 14.6 |
| 5 | 100 | 5 | 7.9 | 17 | 100 | 5 | 14.8 |
| 6 | 100 | 15 | 14.8 | 18 | 100 | 15 | 17.1 |
| 7 | 100 | 25 | 16.7 | 19 | 100 | 25 | 19.5 |
| 8 | 100 | 45 | 20.3 | 20 | 100 | 45 | 26.2 |
| 9 | 150 | 5 | 26.9 | 21 | 150 | 5 | 24.6 |
| 10 | 150 | 15 | 32.9 | 22 | 150 | 15 | 77.6 |
| 11 | 150 | 25 | 70.3 | 23 | 150 | 25 | 94 |
| 12 | 150 | 45 | 95.2 | 24 | 150 | 45 | 98.1 |

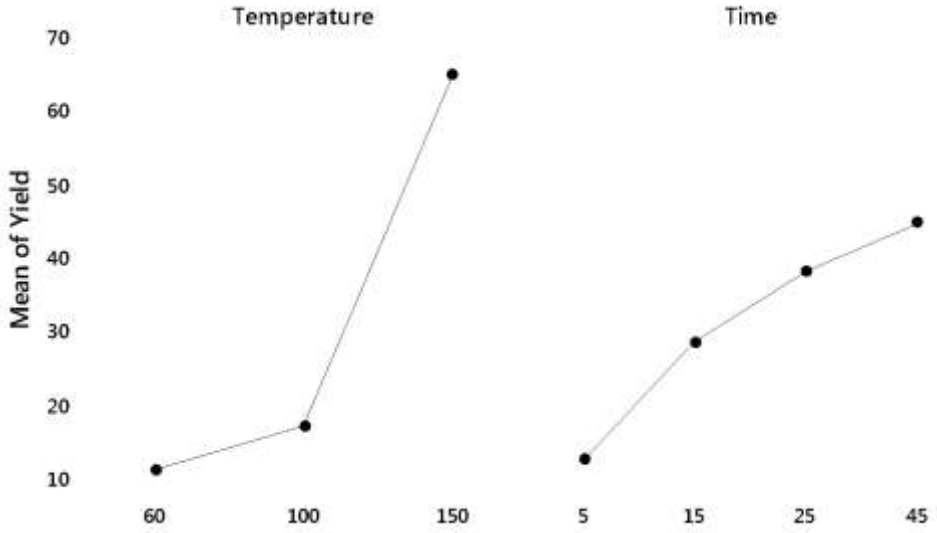



Figure 7.3: Plot of main effect plots of temperature and time towards yield (fixed pH)

Phillippy et al. (1987) studied the hydrolysis of phytic acid as IP6 at different pH levels, using a system at 121 ºC for 1 hour. The hydrolysis yields varied significantly according to pH, and results varied from 45.1% of IP6 degradation to lower inositol phosphates and *myo*-inositol formation at pH 10.8 up to 89.6% of yield at pH 4.0. Based on their study, the lower the pH, the more even distribution of inositol phosphates was obtained. (Chen and Li 2003) studied the degradation of IP6 at 140 ºC with HCl for 1 hour, and obtained a wide range of IP2-IP6 isomers, obtaining decomposition rates of IP6 as high as 95.3%.

Table 7.5 Central composite optimization results of pH and time effect on phytate hydrolysis for fixed temperature = 121 ºC

| Experiment | pH | Time (min) | Yield |
|---|---|---|---|
| 1 | 2.91 | 30 | 47.9 |
| 2 | 4.25 | 30 | 49.3 |
| 3 | 2.91 | 60 | 70.3 |
| 4 | 4.25 | 60 | 94.8 |
| 5 | 2.63 | 45 | 34.2 |
| 6 | 4.53 | 45 | 50.4 |
| 7 | 3.58 | 23.8 | 32.8 |
| 8 | 3.58 | 66.2 | 96.3 |
| 9 | 3.58 | 45 | 100 |
| 10 | 3.58 | 45 | 96.6 |
| 11 | 3.58 | 45 | 99.8 |
| 12 | 3.58 | 45 | 100 |
| 13 | 3.58 | 45 | 95.7 |

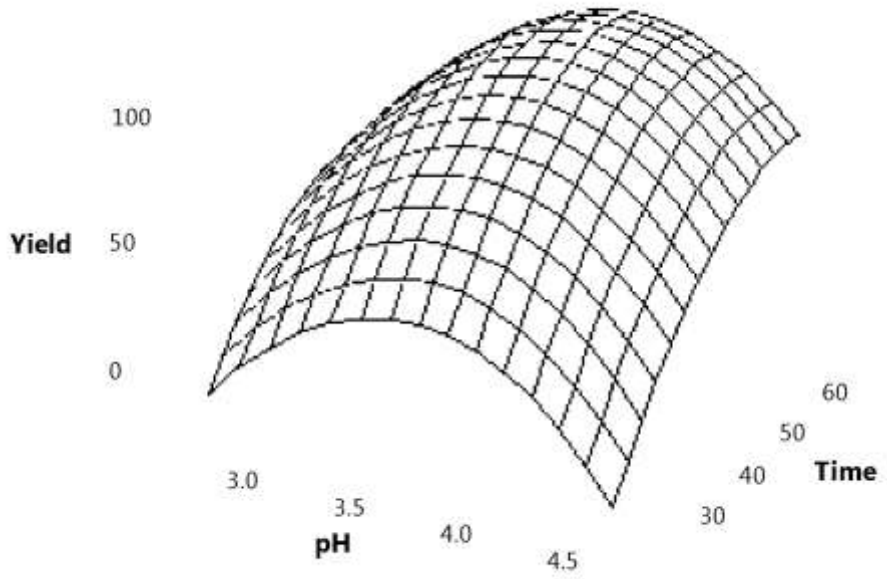



Figure 7.4: Response surface generated from central composite design towards optimization of phytate hydrolysis

### 7.3.3. Environmental profits – removal of P from DDGS

Phosphorus is overdosed in distiller's grains and its removal may positively impact animal feed quality. Removal of phosphorus from animal feed may also infer that manure generated will likely possess less phosphorus, which, will directly have positive environmental impacts, on a sense that less phosphorus present in manure, widely applied as fertilizer, will be applied to soil (Lin et al. 2015). Soils in the Midwest region of the United States are largely overdosed in phosphorus, and these directly impact waterbodies, and lead to algal blooms and eutrophication (Diebel et al. 2009). The technology described in this article is one of the possibilities for removing phosphorus from the precursors of distiller's grains. Other technologies, such as chemical coagulation (Alkan-Ozkaynak et al. 2010) and fungal treatment of thin stillage (Liang et al. 2012) are considered for a simplified environmental-outcome analysis, regarding the potential reduction on total P on DDGS.

Comparison of P extraction processes on DDGS is based on the phosphorus extraction efficiency reported by three categories: (1) phytate extraction from thin stillage via ion exchange, (2) chemical coagulation and removal of phosphorus from thin stillage, (3) fungal cultivation on thin stillage. Phosphorus characterization on ethanol coproducts has been conducted by a number of research groups, and though it is outside of the scope of this article to discuss the phosphorus flow within an ethanol plant, the considerations raised on chapter 3 are maintained: (i) heat does not degrade significantly phytate or total P, (ii) total P is not significantly removed through distiller's corn oil extraction, (iii) P

composition of DDGS can be approximated by contributions of thin stillage and wet distiller's grains. It is also assumed that the addition of chemicals for the technology disclaimed by (2) will not impact the weight of DDGS, thus, mass fractions of each component are relatively unchanged.

Case 1): extraction of phytate from thin stillage using ion exchange

Case 1 relates the operation described on the model developed in this article. (He et al. 2017) described that approximately 0.39 g of P as pure phytate can be extracted from 1 kg of thin stillage under their optimized conditions.

Case 2): addition of chemical coagulants to thin stillage

Chemical treatment processes have low selectivity, thus, the addition of chemical coagulants is likely to produce precipitate formed by organic phosphorus (phytate, and phospholipids) and inorganic phosphorus (phosphates, pyrophosphates, and linear polyphosphates) (Cosgrove, 1980). As described by Liu and Han (2011), inorganic phosphate and phytate represent over 75% of the total phosphorus in thin stillage, thus, chemical treatment methods are expected to result in high removal rates of phosphorus. Phosphates can form a number of precipitate forms with $Ca^{2+}$, such as octa-calcium phosphate, dicalcium phosphate, beta tricalcium phosphate, and hydroxylapatite (Tung, 1998). Alkan-Ozkaynak et al. (2010) reported optimized values to precipitate phosphorus from thin stillage. With a dosage of 66.87 mmol $L^{-1}$ $Ca^{2+,}$ 99% removal of dissolved reactive phosphorus was achieved. Removal of P using Alum was also tested, at the highest dosage of $Al^{3+}$ (19.08 mmol $L^{-1}$), Alkan-

Ozkaynak et al. (2010) observed removal of 66% of total phosphorus. Addition of Aluminumchlorohydrate at 57 mmol $L^{-1}$ resulted in total phosphorus removal of 69.8%.

Case 3) Fungal treatment of thin stillage

Liang et al. (2012) used thin stillage to produce eicosapentaenoic acid (C20:5) by fungal fermentation using *Pythium irregulare*. The C20:5-rich biomass is claimed by Liang et al. (2012) to be an ideal aquafeed that can be used to displace the use of fish oil and fish meal in the aquaculture industry. As an additional benefit of fungal growth in thin stillage, the spent medium, i.e., the culture medium after the fermentation was finished, has significantly decreased amounts of nutrients. The chemical and physical characteristics of thin stillage used by Liang et al. (2012) represent some variation when compared to other research reports, and is likely to be samples derived after a certain degree of evaporation. The solid content of 6.5% and COD concentration of 112 g $L^{-1}$ are slightly higher than those of He et al. (2017) and the summary presented by Reis et al. (2017). The phosphorus removal conditions observed by Liang et al. (2012) are over 93%, achieving a final concentration of 0.11 g $L^{-1}$ in the fungal spent medium.

### 7.3.4. Life cycle assessment on phytate extraction

LCA results indicate that the proposed phytate extraction does not impact significantly most of the environmental impact factors of ethanol production. As a gallon of ethanol was considered as functional unit, the results indicate that for a system with phytate extraction and oil extraction, $CO_2$ emissions will increase from 3.21 kg to 3.28 kg per gallon of ethanol, whilst for a system without distiller's

corn oil extraction, 3.23 kg of $CO_2$ will be increased to 3.30 kg of $CO_2$ per gallon of ethanol produced. NOx, SOx, $N_2O$, and other gases, as seen on table 7.6 also increased with phytate extraction by a factor of about 1%. Table 7.6 represents 4 different cases, (1) with distiller's corn oil extraction and phytate extraction, (2) with distiller's corn oil extraction and without phytate extraction, (3) without oil extraction and without phytate extraction extraction, (4) without oil extraction and with phytate extraction.

Table 7.6: Summary of GREET simulation for cases with and without oil and phytate extraction.

| Emissions | Unit | (1) | (2) | (3) | (4) |
|---|---|---|---|---|---|
| CO2 Total | kg | 3.28 | 3.21 | 3.23 | 3.3 |
| CO2 | kg | 3.28 | 3.21 | 3.23 | 3.3 |
| CO2_Biogenic | kg | -6.99E-04 | -6.51E-04 | -6.27E-04 | -6.75E-04 |
| VOC | g | 2.84 | 2.82 | 2.79 | 2.8 |
| CO | g | 2.99 | 2.95 | 2.93 | 2.98 |
| NOx | g | 6.6 | 6.51 | 6.49 | 6.58 |
| PM10 | g | 1.23 | 1.23 | 1.23 | 1.23 |
| PM2.5 | g | 0.44 | 0.43 | 0.43 | 0.44 |
| SOx | g | 5.97 | 5.88 | 5.86 | 5.94 |
| CH4 | g | 9.66 | 9.42 | 9.5 | 9.75 |
| N2O | g | 2.91 | 2.91 | 2.86 | 2.87 |
| SO2 | mg | 0.45 | 0.45 | 0.43 | 0.43 |
| BC | mg | 46.62 | 45.9 | 45.05 | 45.77 |
| POC | mg | 69.91 | 68.27 | 68.28 | 69.93 |
| Groups | | | | | |
| GHG-100 | kg | 4.36 | 4.28 | 4.29 | 4.37 |
| OnSite Emissions | | | | | |
| CO2 Total | kg | 1.86 | 1.82 | 1.84 | 1.88 |
| CO2 | kg | 1.86 | 1.82 | 1.84 | 1.88 |
| CO2_Biogenic | g | 0.55 | 0.55 | 0.58 | 0.58 |
| VOC | g | 1.8 | 1.8 | 1.77 | 1.78 |

| | | | | | |
|---|---|---|---|---|---|
| CO | g | 0.52 | 0.5 | 0.51 | 0.53 |
| NOx | g | 0.92 | 0.89 | 0.9 | 0.93 |
| PM10 | g | 0.93 | 0.93 | 0.93 | 0.93 |
| PM2.5 | g | 0.22 | 0.22 | 0.22 | 0.22 |
| SOx | g | 0.84 | 0.82 | 0.82 | 0.85 |
| CH4 | mg | 18.77 | 18.13 | 18.24 | 18.89 |
| N2O | mg | 13.14 | 12.8 | 13.02 | 13.37 |
| BC | mg | 12.09 | 11.77 | 11.96 | 12.29 |
| POC | mg | 31.08 | 30.25 | 30.73 | 31.58 |
| Groups | | | | | |
| GHG-100 | kg | 1.87 | 1.83 | 1.85 | 1.89 |
| Flow properties | | | | | |
| Biogenic carbon mass ratio | % | 100 | 100 | 100 | 100 |
| Resources | | | | | |
| Well to Use Resources | MJ | 140 | 139 | 138 | 139 |
| Water Total | m^3 | 0.13 | 0.13 | 0.13 | 0.13 |
| Water_Irrigation | cm^3 | 87341.79 | 87341.79 | 83489.31 | 83489.31 |
| Water_Mining | cm^3 | 32208.35 | 32197.64 | 32085.13 | 32095.92 |
| Water_Process | cm^3 | 11953.77 | 11862.47 | 11844.83 | 11936.15 |
| Water_Cooling | cm^3 | 1102.16 | 1059.22 | 1062.6 | 1105.63 |
| Water_Reservoir Evaporation | cm^3 | 1097.71 | 1054.95 | 1058.32 | 1101.18 |
| Crude Oil | kJ | 2302 | 2281 | 2234 | 2254 |
| Natural Gas | MJ | 34 | 34 | 34 | 35 |
| Coal Average | kJ | 5308 | 5110 | 5141 | 5340 |
| Forest Residue | kJ | 14.7 | 13.67 | 13.14 | 14.18 |
| Pet Coke | J | 4412.88 | 4373.9 | 4283.2 | 4322.25 |
| Corn | MJ | 110 | 110 | 109 | 109 |
| Soybeans | MJ | -14 | -14 | -14 | -14 |
| Renewable (Solar, Hydro, Wind, GeoThermal) | kJ | 18.5 | 17.78 | 17.84 | 18.56 |
| Renewable Natural Gas | J | 7000.91 | 7000.91 | 7361.46 | 7361.46 |
| Uranium Ore | mg | 7.2 | 6.92 | 6.94 | 7.22 |
| Hydroelectric Power | kJ | 236.72 | 227.5 | 228.23 | 237.47 |
| Nuclear Energy | kJ | 760.23 | 730.62 | 732.95 | 762.62 |

| | | | | | |
|---|---|---|---|---|---|
| GeoThermal Power | kJ | 15.89 | 15.27 | 15.32 | 15.94 |
| Solar | kJ | 21.09 | 20.27 | 20.33 | 21.16 |
| Wind Power | kJ | 178.73 | 171.77 | 172.32 | 179.3 |
| Sugarcane | J | 2163.22 | 2163.22 | 2180.48 | 2180.48 |
| Bitumen | kJ | 372.65 | 369.36 | 361.7 | 365 |
| Shale Oil (Bakken) | kJ | 251.78 | 249.55 | 244.38 | 246.61 |
| Shale Oil (Eagle Ford) | kJ | 302.94 | 300.27 | 294.04 | 296.72 |
| Groups | ... | ... | ... | ... | |
| Non Fossil Fuel | MJ | 97 | 97 | 96 | 96 |
| Renewable | MJ | 96 | 96 | 95 | 95 |
| Biomass | MJ | 96 | 96 | 95 | 95 |
| Fossil Fuel | MJ | 43 | 42 | 42 | 43 |
| Natural Gas Fuel | MJ | 34 | 34 | 34 | 35 |
| Coal Fuel | kJ | 5308 | 5110 | 5141 | 5340 |
| Petroleum Fuel | kJ | 3234 | 3205 | 3139 | 3167 |
| Nuclear | kJ | 760.23 | 730.62 | 732.95 | 762.62 |
| Renewable Natural Gas | J | 7000.91 | 7000.91 | 7361.46 | 7361.46 |
| Water | m^3 | 0.13 | 0.13 | 0.13 | 0.13 |
| OnSite Resources | MJ | 122 | 122 | 121 | 122 |
| Conventional Diesel | kJ | -397.91 | -397.91 | -418.4 | -418.4 |
| Diesel For Non Road Applications (High sulfur content) | kJ | -12.52 | -12.52 | -13.16 | -13.16 |
| Residual Oil | kJ | -13.7 | -13.7 | -14.41 | -14.41 |
| Bunker Fuel | kJ | -20.01 | -20.01 | -21.04 | -21.04 |
| Natural Gas | MJ | 23 | 22 | 23 | 23 |
| Coal Average | kJ | 1688 | 1638 | 1657 | 1708 |
| Forest Residue | kJ | -11.86 | -11.86 | -12.47 | -12.47 |
| Electricity | kJ | 2548 | 2482 | 2499 | 2565 |

## 7.4. Conclusions

The removal of phytate from thin stillage may represent a novel process in the dry-grind ethanol industry. It was shown in this chapter that the removal of phytate via ion exchange does not increase significantly (around 1% of increase) the emissions involved on the production of one gallon of ethanol, and that it can be a source of profit if operated within a certain range of thin stillage processed within a plant. The results also presented herein describe the potential of converting the extracted phytate to myo-inositol. Myo-inositol has a more flexible market when compared to phytate, and could also represent another source of revenue for ethanol plants.

# CHAPTER 8. GENERAL DISCUSSION AND PROPOSED WORK

## 8.1. Research motivation on phytate extraction

Minnesota is the third largest corn and corn ethanol producer in the United States, producing over 1 billion gallons annually. The addition of a high-value stream production by these plants will have a direct impact on their economic performance. The preliminary modeling using techno-economic models suggest favorable returns for entering the market for phytate extraction and sales of proper quality grades to serve nutritional and metal treatment markets. The improved competitiveness of ethanol plants implementing the technology presented in this dissertation will be able to create jobs and increase ethanol profitability by creating a product set to meet an existing market demand. Currently, China and Japan are the leading producers for the vast majority of phytate produced and commercialized in the world, and American industries rely vastly on this product. Having domestic producers would greatly enhance the local economy by establishing competitive and high-quality market.

Currently, the main co-product from dry-grind ethanol companies is DDGS. Production of DDGS in North America has now surpassed that of soybean meal in terms of tonnage Therefore, DDGS and sales of DDGS to pork producers represent a significant source of revenue to ethanol plants and multiple trading and commercialization companies in Minnesota. However, not all value of DDGS is captured by pork producers. Typically, DDGS contains high concentrations of indigestible phosphorus and when used in diets for growing pigs, this excess

phosphorus is excreted into the environment. Phytate extraction from the ethanol coproducts streams will remove the indigestible portion of phosphorus from DDGS, resulting in decreased phosphorus excretion in manure In addition, Phytate has a strong chelating capacity, thereby reducing digestibility of other nutrients such as amino acids and it is often seen as an anti-nutritional factor for monogastric animals, like poultry and swine. By making the formerly indigestible phosphorous available from DDGS for the monogastric livestock, livestock producers can reduce the inclusion of other sources of phosphorus in the diets. It is widely known that a significant factor in eutrophication in the Midwest is due to overdosed amounts of applied phosphorus from commercial fertilizers and manure. Therefore, reducing phytate content from DDGS may enhance manure properties and decrease its adverse effects to the environment. Producing DDGS with reduced phytate will help sustain the market demand for reduced-oil DDGS and sustain DDGS sales from ethanol plants, possibly even increasing its market value.

Furthermore, phytate extraction in ethanol plants will require labor force, thus, creating jobs and increasing ethanol profitability. Phytate extraction from corn ethanol coproducts will reduce the amount of indigestible phosphorus in DDGS, which is fed to food producing animals. Conversion of phytate to inositol will also provide another source of a valuable nutrient for human and animal health, which will also be produced locally. Inositol may be a more attractive product than phytate in the long-run, depending on costs of refinement and markets.

The implementation of phytate extraction technology in commercial plants in Minnesota, as well as inositol production, would create an additional revenue stream from these dry-grind ethanol plants. It will give them a distinct economic and technical advantage towards other ethanol plants in the country. It will create new jobs and require marketing and logistics mechanisms for distribution of phytate and inositol.

## 8.2. Background information on phosphorus

### 8.2.1. Commercial phosphorus production

Commercially available phosphorus is mostly produced by using calcium apatite, $Ca_5(PO_4)_3OH/F$, which is granulated and a binder is added on a rotating dish. From this operation, pellets are obtained and dried at high temperatures. Rock pellets are mixed with cokes and silica and fed into a furnace. This process is able to reduce the phosphate present in the slurry material to $P_4$, leaving the furnace as a gas, with carbon monoxide and dust as by-products. Dust is removed using a precipitator and it is recycled into the process. $P_4$ is condensed, and CO is used as a fuel for the sintering plant. The other residual products, which are mostly composed by silica and calcium oxide are cooled and pelletized. This coproduct is often used in road construction and low-quality cement. The recycling process adds phosphorus residuals to the rock before the initial grinding stage, achieving above 90% of recovery rates (Morse et al. 1998).

### 8.2.2. Phosphorus organic cycle

There are two organic cycles that move phosphorus through living organisms as part of the food chain. The first is a land-based cycle that transfer it from soil to plants and animals, and back to the soil. The second cycle is a water-based

organic process that circulates among creatures living in waterbodies, such as rivers, lakes, and seas. The land-based cycle takes a year on average, and the water-based lasts for a few weeks.

The amount of phosphorus in the world's soils is estimated to be 2 giga Tons, however a small fraction is available to biota in most soils (Abelson 1999). Even greater values are found in oceans, which is fractionated dissolved in water and in sediments, mostly as calcium phosphate. The use of phosphates to nourish agricultural soils has the objective of replenishing the removal of phosphorus from soil by harvests and erosion losses. Due to this, application of P ranges in values of 3 kg P $ha^{-1}$ in South Africa to over 34 kg $ha^{-1}$ in Ireland (Driver et al. 1999).

### 8.2.3. Phosphorus in manure: an important secondary source

In regions with intensive livestock agriculture, animal manure storage and disposal can pose a large environmental problem. In most cases, the production of manure exceeds the nutrient requirements in the lad, which, lead to excess fertilization when applied as fertilizer. Throughout the last decades, several projects have been diagnosing nutrient recycling in manure. Most of them include drying the manure or concentrating it to facilitate its transportation to areas where the nutrient limits are not reached yet.

The phosphate present in manure can also be used as a raw material for the P industry. In untreated manure, the levels of organic matter and water are unbalanced to the low phosphate concentration, making the process cost inhibitive. Several researchers have proposed an incineration to be used in the P

industry. A project developed by Dutch researchers studied the incineration of air-dried manure, with conversion of produced energy to electricity. The end product, a concentrated calcium phosphate powder, has been used successfully (Driver et al. 1999).

### 8.3. A brief between sugarcane and corn ethanol technologies

The traditional sugarcane-to-ethanol process in Brazil lies back to the 1980's, posing Brazil as a top producer and role model for decades. Recently, the United States surpassed the Brazilian numbers and today account for the highest volume of ethanol produced in the world, accounting for over half of the world production. Taking in account that the main product in an ethanol plant is ethanol *per se*, it is challenging to think that a more complex reaction system to produce the same compound can correspond to the state-of-art production system. The sugarcane ethanol is produced through fermentation of sugarcane juice, i.e., a solution with readily available sugars to yeast, mostly composed by sucrose, while corn, the main feedstock for ethanol production in the United States, is a feedstock material with higher complexity than sugarcane. Corn is a rich substrate for most biological systems, rich in proteins, starch, lipids, and with enough minerals to convey and support microbial growth. Sugarcane, on the other hand, is a simple, yet, effective feedstock for ethanol production. Sugarcane does not have significant quantities of lipids, complex sugars in its juice, and is deficient in minerals, such as phosphorus and nitrogen. Sugarcane by itself would not be a suitable fermentation feedstock for most industrial applications without added nutrients. It is, however, extremely suitable for ethanol

fermentation via yeast due to a number of factors: (i) simple fermentable sugars that do not require saccharification, and consequential addition of exogenous enzymes, e.g., alpha-amylases, (ii) nitrogen deficiency promotes cell stress that inhibit cell growth, promoting most of the carbon flux go through ethanol production instead of the undesirable cell growth, (iii) rheological properties that allow a fast nutrient transfer from the media to the yeast. Corn when processed for ethanol production, accounting as of today for over 40% the farmable numbers for its production, especially when processed through the dry-grind process, that provides a viscous mash with all the corn kernel components grinded to a saccharified and liquefied mass, as fermentation material possess opposite characteristics as those pointed for sugarcane. Yet, against the technical odds, dry-grind corn ethanol production accounts for over 90% of the corn-to-ethanol production. The dry-grind ethanol industry is living its golden age, and is growing in production and capacity over the past years, and it is still projected to continue growing. Sugarcane juice is usually fermented to its maximum potential within 6 to 10 hours in an average ethanol plant, while corn through the dry-grind process has a retention time in a fermenter from 48 to 72 hours. Sugarcane is harvested in Brazil 3-4 times a year, and present today higher plantation and harvesting yields than those in the 1980’s, while corn is still harvested once. Yet, sugarcane lies behind corn in total volume processed for ethanol production. The answer to such an intriguing question involves political, social, and technical issues. The latter are discussed within the appendices, and

are commented on being on the underdevelopment or underperformance of Brazilian sugarcane-to-ethanol industries in utilizing the residuals of the fermentation, i.e., the by-products. By-products are no longer a term accepted by the dry-grind corn-to-ethanol industry, and rather are named coproducts. The change in upgrading secondary products, processing, and marketing them to another industrial sector, such as the feeding industry, is an underdeveloped task in the sugarcane-to-ethanol process that can, and should, be changed.

### 8.4. Proposed future work

It is proposed that researchers keep evaluating and optimizing the phytate extraction system presented in this dissertation. The results are constrained to industrial-grade anion exchange resins, which could be potentially modified for enhanced performance. Since up to the moment of this dissertation, no actual industrial demonstration was performed, uncertaintity of the robustness of the proposed work is still a concern. As the technology has been patented by Dr. Hu's group, future applications of phytate extraction in dry-grind corn-to-ethanol plants is a possibility in discussion.

It is also strongly suggested that the scope of this research does not restrain itself only to dry-grind corn-to-ethanol plants. The massive scale of ethanol plants (exemplified in numbers on chapters 3 and 7) represent the target market of the phytate extraction technology. However, other distilleries that mimic the corn-to-ethanol dry-grind process, such as those using wheat, rye, and other grains, could benefit of this technology. Examples such as whisky distilleries, since they are smaller in size, could potentially be a source of phytate in the market.

Though the main focus of this work being related to phosphorus capture and utilization, it is also urged that similar work to be done with nitrogen in ethanol coproducts. Understanding protein flow in ethanol coproducts is still a research question, and research reports and literature data differ significantly when it comes, for example, to the contribution of yeast protein towards total protein of DDGS. Opportunities for application of nitrogen remediation and recovery of nutrients can, thus, be developed upon better understanding of protein and nitrogen flow in ethanol coproducts.

A strong suggestion for future work is regarding thin stillage filtration. This topic was not studied in depth during the time course of this dissertation for a few reasons: (i) due to a patented technology that is in pilot demonstration by a large player in the ethanol market in the United States, (ii) being out of the scope of the grant proposal from MnDRIVE to which the project of phytate extraction was funded, (iii) the complexity of thin stillage filtration, with unusual cake filter behavior observed in preliminary studies, (iv) the difference in thin stillage within batch to batch and from company to company – which could lead to a solution to one single company, i.e., a system development without the required robustness for this kind of industry, and (v) due to the filtration of thin stillage having been evaluated by other research groups, especially by a renowned group at the University of Illinois at Urbana-Champaign.

The review provided on chapter 2 in this dissertation shows the wide variety of technological options developed upon the use of thin stillage. Many of those

options are still under development and are not represented in the industrial scale. Scalability of processes using thin stillage as major feedstock is, thus, a suggested approach to be taken by future research groups.

Chapter 8 provides a preliminary approach to the use of artificial lichens on whole stillage. The use of bioprocessing in ethanol coproducts is a realm of opportunities, and due to the flexibility of microorganisms, many options are available for production, accumulation, extraction, and purification of high-value compounds derived from ethanol coproducts.

Due to the importance of the ethanol industry in the United States and Brazil, the social, ecological, environmental, and the all the facets of ethanol production and implications towards societal development are always a possibility of studies. Though being outside of the scope of this dissertation, understanding that engineering upgrades in any industry can have direct causation towards people in general is key for a sustainable future.

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# APPENDIX 1. ARTIFICIAL LICHENS AS AN ALTERNATIVE TO INCREASE FEEDING VALUE OF DISTILLER'S GRAINS

**Outline**

Objective: To increase the linoleic acid content in distiller's grains and decrease the excess nutrients in stillage water by the use of an artificial lichen, composed of fungi, algae, and a supporting matrix.

Results: A maximum concentration of 46.25% of linoleic acid in distiller's grains was achieved with a combination of *Mucor indicus* and *Chlorella vulgaris* using corn-to-ethanol whole stillage as substrate. Microbial hydrolytic enzymes during the fermentation were able to decrease the solids in whole stillage. N depletion by microalgal uptake causes lipid-formation stress to *Mucor indicus* cells, increasing linoleic acid production to about 49% of the total lipids, potentially decreasing costs in animal feed.

Conclusion: Supernatant can potentially be recycled as process water to ethanol fermentation tank, and enhanced distiller's grains can replace animal-specific diets. This would reduce exogenous enzyme use and supplementation of unsaturated fatty acids from other sources.

**Introduction**

The use of distiller's grains in animal feed formulation has been a factor of success to dry-grind corn-to-ethanol plants over the past 20 years, with annual production capacity in the order of 40 metric tons a year (Reis et al. 2017). Distiller's grains are produced using the residues of the fermentation material that is processed into grain-to-ethanol fermentation facilities, briefly (figure 1.a). A few key issues still stand out as challenges for the ethanol industry, including water usage and recyclability, and higher displacement of distiller's grains in animal feed rations. The use of distiller's grains in animal use have animal-specific

challenges, e.g., for dairy cattle a lack of linoleic acid, key fatty acid regulating milk fat (Chilliard et al. 2000), is observed, while for monogastric animals, proteins and phosphorus have limited digestibility (Lupitskyy et al. 2015). Anaerobic digestion of stillage water is currently used to decrease nutrient complexity, providing an alternative for water recycling in ethanol plants, achieving commercial rates of up to 58%, though encountering technical difficulties due to the high presence of suspended solids, and imbalance of C:N, often requiring higher processing times, and not being a practice exploited to its full potential (Reis et al. 2017).

The use of secondary fermentation of ethanol coproducts has been reported as a way to produce malic acid (West 2011), ethanol (Gonzalez et al. 2010), and a significant number of chemicals (Reis et al. 2017). Few studies, however, focus on improving the animal feed quality by fermentation. The purpose of the current article is to present a bioprocess platform developed using whole stillage, the bottom fraction of ethanol distillation, rectifying, and stripping operations. The use of an artificial lichen biofilm, composed by fungi, algae, and a supporting matrix, reported as *mycoalgae*, was chosen due to the potential of algae and fungi to uptake nutrients (Rajendran et al. 2017) and produce hydrolytic enzymes (Gutierrez-Correa and Tengerdy 1997), as a way to replace costly anaerobic digestion practices, and evaporation of stillage water. Simultaneously, nutrients from whole stillage are processed into the mycoalgae biomass, providing an increase in lipids and production of linoleic acid, as depicted in figure 1.b.

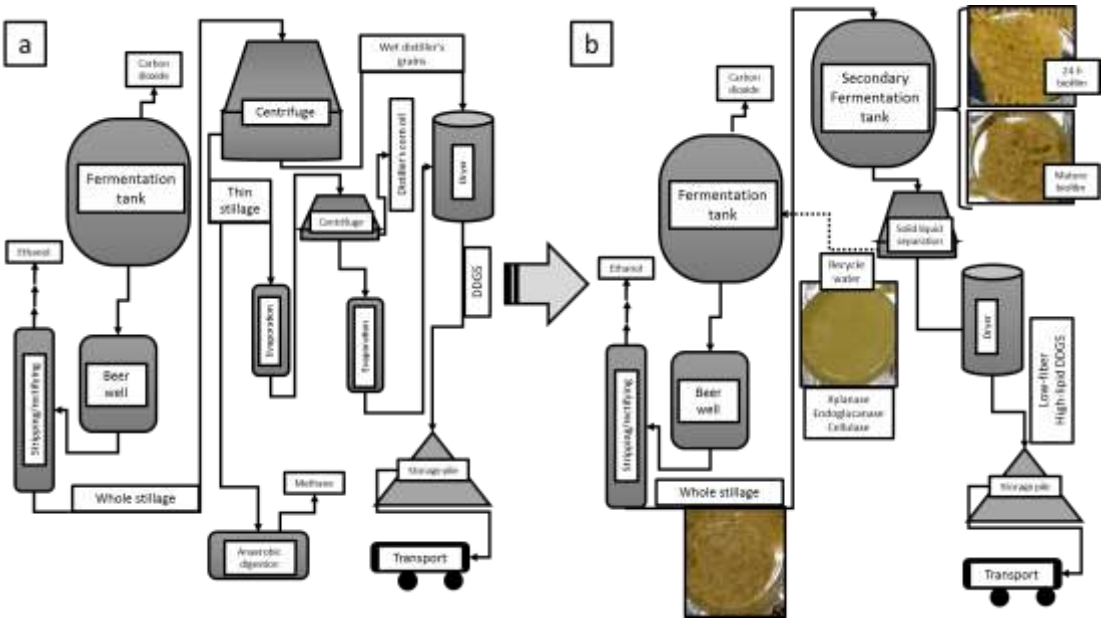


Figure A1.1: a) traditional downstream processing in dry-grind ethanol plants; b) potential modification using mycoalgae to increase value of DDGS

**Materials and methods**

**Microorganisms, medium preparation, and experimental design**

*Chlorella vulgaris* 2714 (UTEX) and *Mucor indicus* (ATCC) were chosen as model biofilm-forming strains, and were maintained in medium A (Rajendran and Hu 2016). Whole stillage (WS) was collected from a dry-grind ethanol plant in the state of Iowa, USA. WS samples were stored at -20 ºC prior to use, and were used for medium prepared without solid-liquid separation or dilution. Fermentation experiments were prepared according to Rajendran and Hu (2016). The medium pH was adjusted to different values as dictated by the experimental design by addition of 2 mol HCl $L^{-1}$ or 2 mol NaOH $L^{-1}$ before autoclaving. The culture medium was inoculated with the co-cultures of *M. indicus* fungal spores and *C. vulgaris* algae cells at a ratio of 1:300, with an initial algae count of

2.50E9 cells, unless specified differently. Two major set of experiments were performed in the current study: a kinetic study, with the control conditions above, and periodic samples, and a Taguchi $L_9$ array, evaluating initial pH, temperature, and algae:fungi inoculation ratio, each in 3 levels. Each condition was performed with triplicates for mycoalgae, 2 algae controls, 2 fungi controls, and 1 blank flask. Statistical analysis was performed using Minitab® 17.

**Cell harvest and analytical methods**

Cell separation, and all measurements regarding cell biomass were based on Rajendran and Hu (2016). Total phosphorus (TP), nitrogen (TN) and chemical oxygen demand (COD) in the culture liquid were measured using Hach TNTplus® analysis kits (TNT 845, TNT 827, and TNT 822, respectively). For lipid analysis, biofilm and the fungal biomass were oven-dried until constant weight at 105 °C, and processed according to Bligh-Dyer method. Lipid content was determined by calculating the weight percentage of lipid in dry biomass, and lipid profile was measured using GC-FID (Reis et al. 2014). Enzymatic activities were estimated using timed hydrolytic activities on pure standard substrates, and 1 U was defined as the amount required to generate 1 μmol of substrate per minute of reaction.

**Results and discussion**

**Mycoalgae development in whole stillage**

*Chlorella vulgaris* and *Mucor indicus* were inoculated in Erlenmeyer flasks containing whole stillage and a presence of an attachment matrix. It has been reported by Rajendran and Hu (2016) that in culture systems with axenic algae and fungi cultures, fungi completely attach to the matrix material, while algae

grow in suspension, similarly observed in this study. On a co-culture system, however, algae attach to the fungal mycelia, which is retained at the matrix. Fungal cell growth occurred on a similar process as described by Rajendran and Hu (2016), with fungal mycelia initially developing on the mesh material, with later algae attachment. Leveraging the use of whole stillage for fermentation may positively impact the whole coproducts processing, potentially decreasing dry-grind process complexity. *M. indicus* is generally-regarded-as-safe (GRAS) and *C. vulgaris* has beneficial effects if included in animal feed at optimal levels (Spolaore et al. 2006).

**Cell growth – kinetics and biomass distribution**

The assumption used in this work was that whole stillage was a feedstock material rich enough to sustain microbial growth, and despite having complex macromolecules, such as proteins and polysaccharides derived from corn and yeast, it could still promote a significant fungal growth given enough oxygenation, potentially increased by algae cultivation. While the total biomass present in the biofilm was maximum at around 96 h (10.7 g $L^{-1}$) of the process, it decreases to 8.9 g $L^{-1}$ at 120 h. The fungal and algal components in the biomass either plateaued or grew during the entire cultivation time, indicating a loss in attached solids in the biofilm. The total attached solids in the mycoalgae biofilm decreased from 3.15 g $L^{-1}$ in the first day of cultivation to around 1 g $L^{-1}$ after 120 h of growth, possibly caused by the production of hydrolytic enzymes by the fungi and algae present in the biofilm. Results are in consonance with those presented by Rajendran et al. (2017), indicating an increase in microbial biomass with

concurrent decrease in the solid content as the process time increases. Compositional analysis of the biofilm indicate that the microbial biomass constitute 6.6% of total mass attached at 24 h of growth, and after 120 h, it increases to 88.7% of the total biomass present in the biofilm comprising of algae and fungi (Figure 8.2).

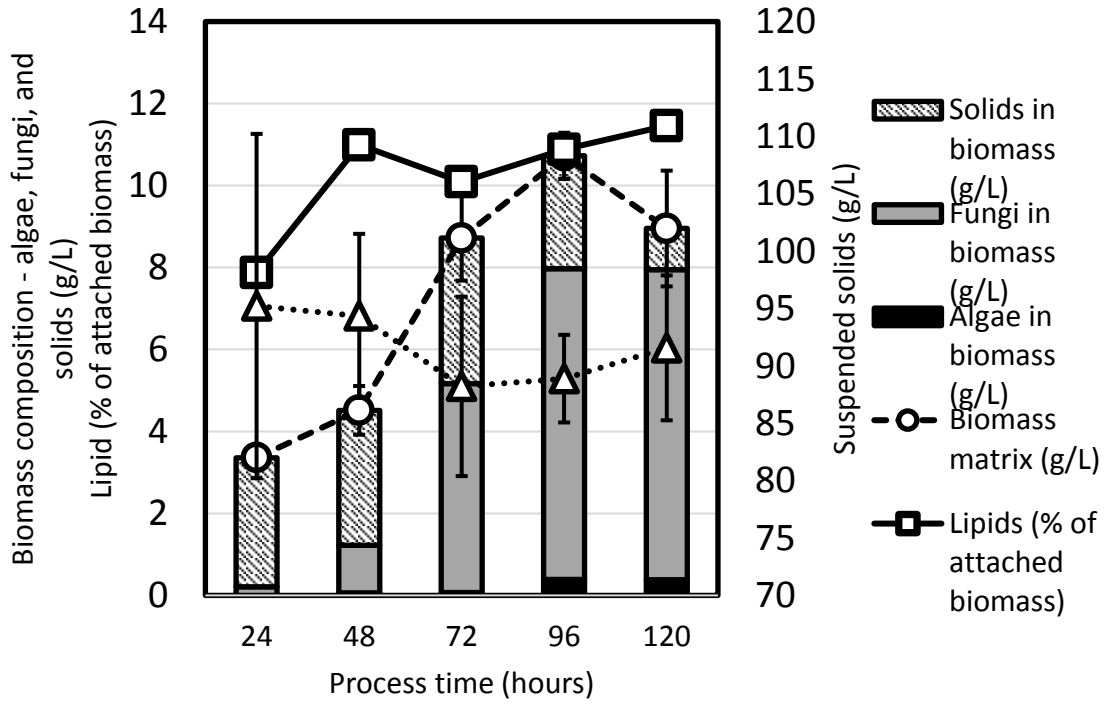


Figure A1.2.: Kinetic profile of mycoalgae growth in whole stillage

In whole stillage co-culture process, algae biomass contributes to about 5% in the mature biofilm, higher than the 3% reported by Rajendran et al. (2017) on distiller's solubles. The hypothesis for an increase in microalgae concentration in whole stillage is that the high turbidity of the complex medium posed difficulty for a purely photoautotrophic growth. It is, thus, believed that the consortium of

algae and fungi hydrolyzed organic carbon sources present in whole stillage to sustain algae growth in a heterotrophic system.

**Factorial results**

**Nutrient removal and enzymatic activity**

As one of the objectives of this study is to evaluate the enzyme fortified water reusability in the ethanol industry by employing the fermentation of mycoalgae in whole stillage, the removal of nutrients from the supernatant phase is, thus, a desired outcome. The composition of whole stillage showed a COD value of about 80.25 g $O_2$ $L^{-1}$. Initial nitrogen concentration was of 1250 mg $L^{-1}$, mostly composed by dissolved proteins, amino acids, and residues of ammonia from the corn-to-ethanol fermentation. Previous research indicates *C. vulgaris* as a microorganism with N removal potential, with kinetics of N uptake greater than those of fungal activities (Rajendran et al. 2017). The presence of high levels of P has been a concern in ethanol coproducts, especially due to its concentration in distiller's grains, which may not be fully degraded by animals. Initial concentration of total phosphorus present in the whole stillage medium was 1380 mg $L^{-1}$, which was within expected range based on published literature results. The $L_9$ results on table 1 indicate a maximum COD and N removal of about 52% and 82%, respectively by the mycoalgae biofilm. P removal on all conditions tested using the mycoalgae biofilm was greater than 50%.

The chemical composition of thin stillage is a critical factor that limits the use of such substrate in anaerobic digestion for water recycling. In addition to removing nutrients, conditioning the reused water for further use in the upstream and

fermentation steps is economically desirable. Aerobic fermentation can, thus, be an alternative to anaerobic digestion. Among the classes of enzymes used in the corn-to-ethanol process, starch-hydrolyzing enzymes are the key regulator in the fermentation of corn, nonetheless, current utilization of different classes of protease, phytase and fiber-degrading enzymes has been reported. Therefore, the goal of recycling water within the ethanol production system, simultaneously providing a suspension with hydrolytic enzymes, was evaluated, in order to potentially integrate downstream water to upstream processing of posterior batches. The maximum production of endoglucanase, xylanase, and cellulase was observed on the same experiment, and ranged from 30.77 to 38.69 U $mL^{-1}$. From the factorial analysis, initial pH was the most significant factor, and the optimum value among those tested is the closest to neutral, pH = 6.8. Within the mesophilic range studied (27-33 ºC), enzymatic production was robust, and indicated that temperature fluctuations under the mesophilic range will not likely impact the enzymatic production. Initial fungi:algae ratio is not significant at a 5%-confidence level, however, there is a trend when increasing the algae population in the mycoalgae to produce greater amounts of cellulase, endoglucanase, and xylanase. As an example, using initial pH = 6.8, xylanase production increased from 29.40±4.18 U $mL^{-1}$ to 38.69±5.51 U $mL^{-1}$ when the initial algae population was 4 times greater, i.e., comparing results for 1:300 and 1:1200 inoculation ratio, respectively.

**Lipid analysis**

Corn-to-ethanol units have been recovering distiller's corn oil from the liquid phase of whole stillage, i.e., thin stillage. Despite being of significant economic advantage for ethanol producers, DDGS derived from processes with extraction of distiller's corn oil is often reported to have inferior nutritional properties when compared to full-fat DDGS (Reis et al. 2017). Several strains of *Mucor sp*. fungi have been reported as a lipid-producing and accumulating microorganisms (Ratledge 2002). It is known that fungal bodies begin accumulating lipids by the depletion of a nutrient, usually N (Ratledge 2002) - WS has a COD:N ratio of 64.2, thus being a N deficient medium, with potential to be used with lipid-producing strains. A current concern in distiller's grains utilization for dairy cattle is the lack of linoleic acid. Linoleic acid deficiency has been linked as one of the most significant causes for milk fat depression, a condition in which the lipid content of milk from cattle is decreased (Chilliard et al. 2000). Statistical analysis indicate the factor with the greatest significance on lipid accumulation to be the initial pH of the medium. Previous research prove that initial pH regulates lipid accumulation by fungi (Xia et al. 2011). From a Pearson correlation between final total N concentration in the supernatant and lipid accumulation (results from table 1) it is statistically proven (p-value = 0.017) that the increase in lipid accumulation is regulated by the final N concentration in the suspension phase. On regards to linoleic acid accumulation, a maximum of 46.25% was achieved, having as factor with the greatest significance the initial pH of the medium. The presence of the algae in the biofilm formation increased the lipid quantity and the linoleic acid

accumulation in all the experiments. The hypothesis behind this experimental fact is due to the high rates of N uptake by microalgae (Rajendran et al. 2017), which caused additional stress, causing the fungi to accumulate lipids.

Table A1.1: L9 experimental results. Bottom of table accounts for factorial analysis given as F statistics for a given factor and a given response and p-value in parentheses.

| $pH_0$ | Temp. (ºC) | fungi:algae (spores:cells) | Xylanase activity (U $mL^{-1}$) | Endoglucanase activity (U $mL^{-1}$) | Cellulase activity (U $mL^{-1}$) | Lipid (%) | C18:2 (% lipid) | $TN_f$ (mg $L^{-1}$) | $TP_f$ (mg $L^{-1}$) | $COD_f$ (g $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| 4.6 | 27 | 1/1200 | 23.69±0.50 | 23.23±0.14 | 20.22±0.47 | 4.03±1.71 | 11.78±1.04 | 823.33±77.78 | 614.44±9.55 | 44.93±6.76 |
| 4.6 | 30 | 1/600 | 23.59±5.15 | 23.37±0.16 | 20.43±0.27 | 6.38±2.62 | 7.49±0.86 | 473.00±116.37 | 493.33±47.13 | 32.15±4.19 |
| 4.6 | 33 | 1/300 | 23.80±0.95 | 23.80±0.49 | 20.62±0.93 | 5.89±0.96 | 7.33±3.87 | 482.62±15.79 | 599.09±22.01 | 36.89±11.14 |
| 5.7 | 27 | 1/600 | 32.34±3.74 | 25.49±1.01 | 24.68±2.41 | 9.49±0.97 | 9.72±1.58 | 525.21±71.37 | 616.79±58.29 | 39.03±8.52 |
| 5.7 | 30 | 1/300 | 25.45±2.57 | 23.55±1.44 | 20.68±3.35 | 6.10±0.83 | 7.46±2.60 | 371.17±41.37 | 622.90±40.18 | 28.55±4.53 |
| 5.7 | 33 | 1/1200 | 37.58±2.14 | 29.76±1.74 | 30.64±1.79 | 9.33±1.97 | 6.48±0.87 | 166.67±38.18 | 585.62±29.14 | 31.49±10.96 |
| 6.8 | 27 | 1/300 | 29.40±4.18 | 24.77±1.88 | 23.97±2.92 | 8.74±5.66 | 38.98±0.59 | 194.33±61.52 | 584.36±34.49 | 37.17±3.14 |
| 6.8 | 30 | 1/1200 | 38.69±5.51 | 31.88±3.66 | 30.77±3.99 | 12.08±1.68 | 32.62±0.68 | 261.02±4.95 | 550.52±18.62 | 32.27±1.76 |
| 6.8 | 33 | 1/600 | 37.94±3.14 | 31.16±1.26 | 30.49±2.01 | 14.98±3.30 | 46.25±1.65 | 111.73±69.86 | 559.77±18.79 | 41.61±5.40 |

| Factor | Xylanase activity (U $mL^{-1}$) | Endoglucanase activity (U $mL^{-1}$) | Cellulase activity (U $mL^{-1}$) | Lipid (%) | C18:2 (% lipid) | $TN_f$ (mg $L^{-1}$) | $TP_f$ (mg $L^{-1}$) | $COD_f$ (g $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| $pH_0$ | 21.29 (0.045) | 21.55 (0.044) | 24.15 (0.040) | 37.92 (0.026) | 41.16 (0.024) | 5.39 (0.157) | 1.06 (0.485) | 1.22 (0.450) |
| Temperature | 3.68 (0.214) | 8.98 (0.100) | 7.51 (0.117) | 6.65 (0.131) | 0.77 (0.564) | 2.23 (0.309) | 1.13 (0.470) | 3.95 (0.202) |
| Algae:fungi | 8.00 (0.111) | 11.76 (0.078) | 11.32 (0.081) | 10.18 (0.089) | 0.62 (0.616) | 0.16 (0.864) | 0.96 (0.511) | 0.51 (0.661) |

# APPENDIX 2: VINASSE FROM SUGARCANE ETHANOL PRODUCTION: BETTER TREATMENT OR BETTER UTILIZATION?

**Outline**

Ethanol production from sugarcane in Brazil is a well-established industry, with relatively simple operations and high yield. The ethanol primarily serves as a renewable fuel to with gasoline and diesel to increase the energy security in Brazil. Several environmental concerns are emerged around the by-products from this industry. Vinasse, the liquid fraction generated from the rectifying and distilling operations of ethanol, is a sulfur-rich, low pH, dark-colored, and odorous effluent, and it is produced at volumes as high as 20 fold of ethanol. Traditional wastewater treatments, such as bioprocessing, advanced oxidative processes, anaerobic digestion, and chemical-based processes, have been applied to vinasse management. Despite most of its utilization being in fertirrigation practices, vinasse may represent a key factor in enhancing profitability and environmental outcomes of a sugarcane-to-ethanol plant. The application of some upgrade solutions to sugarcane-derived vinasse may represent additional sources of energy, production of animal feed components, and reduction in water consumption within a plant. This mini-review article provides a critical and comprehensive summary of the alternatives developed or under development to vinasse management.

Keywords: vinasse, sugarcane, anaerobic digestion, fertirrigation, ethanol

## Introduction

Brazil is home to over 300 active sugarcane biorefineries (Filoso et al., 2015), with an ethanol-rich history dating back to the 1970's (Goldemberg et al., 2008). The oil embargo crises over 40 years ago forced the Brazilian government to find alternative solutions for energy generation. The most successful strategy was the National Ethanol Program (Proálcool), which increased Brazil's energy security and posed it as the largest ethanol producers for decades (Goldemberg et al., 2008). Even with recent global booming of ethanol and biofuel industry, Brazil is still the second largest ethanol producers in the world, summing values of 25 billion L per year (Walter et al., 2011).

Over 75% of the Brazilian distilleries operate using the Melle-Boinot process, a fed-batch system using yeast cell recycling. Yeast cells are collected at the end of the fermentation cycle, and are either centrifuged or filtered, and re-inoculated to the next fermentation cycle (Brethauer and Wyman, 2010). The high-density cell culture and simple composition of sugarcane juice allow a quick fermentation cycle (6-10 h), and low cell growth rates (Della-Bianca et al., 2013). The upstream steps include a sulfitation process, which enrich the downstream products with sulfur compounds, especially sulfate species (Della-Bianca et al., 2013). After fermentation, the fermented juice is processed into an ethanol stream and a liquid-rich by-product – vinasse.

Sugarcane vinasse is a residue from the sugar-ethanol industry, characterized as being an acidic suspension, high COD values, unpleasant odors and dark brown color (Gómez and Rodríguez, 2000; Jiang et al., 2012; Christofoletti et al., 2013).

The characteristics of vinasse are largely dependent on the feedstock, and on the fermentation and distillation conditions applied (España-Gamboa et al., 2011). A summary of vinasse composition is presented on table 9.1.

Table A2.1.: Composition of sugarcane vinasse

| Component | Value | Reference | Component | Value | Ref. |
|---|---|---|---|---|---|
| **Mineral analysis** | | | **Organic analysis** | | |
| $Cl^-$ (mg $L^{-1}$) | 59.4 | dos Santos et al. (2013) | Organic matter (%) | 3.96 | Mariano et al. (2009) |
| $SO4^{2-}$ (mg $L^{-1}$) | 1680 | dos Santos et al. (2013) | C:N ratio | 10 | Mariano et al. (2009) |
| $Na^+$ (mg $L^{-1}$) | 8.6 | dos Santos et al. (2013) | COD (mg $L^{-1}$) | 32000-92800 | Mariano et al. (2009), Paz-Pino et al. (2014) |
| $K^+$ (mg $L^{-1}$) | 1620 | dos Santos et al. (2013) | $BOD_5$($mgO_2$ $L^{-1}$) | 13514-36847 | Paz-Pino et al. (2014) |
| $Ca^{2+}$ (mg $L^{-1}$) | 3160 | dos Santos et al. (2013) | $BOD_5$/COD | 0.18-0.34 | Paz-Pino et al. (2014) |
| $Mg^{2+}$(mg $L^{-1}$) | 162.4 | dos Santos et al. (2013) | Phenols (mg $L^{-1}$) | 230-390 | Paz-Pino et al. (2014) |
| $PO4^{3-}$ (mg $L^{-1}$) | 560 | dos Santos et al. (2013) | $NH_4^+$ (mg $L^{-1}$) | 23.9 | dos Santos et al. (2013) |
| $NO_3^-$ (mg $L^{-1}$) | 823.7 | dos Santos et al. (2013) | protein (%) | 2.92 | Dowd et al. (1994) |
| Fe (mg $L^{-1}$) | 44.9 | dos Santos et al. (2013) | fiber (%) | 0.2 | Dowd et al. (1994) |
| Mn (mg $L^{-1}$) | 4.9 | dos Santos et al. (2013) | fat (%) | 0.41 | Dowd et al. (1994) |
| Zn (mg $L^{-1}$) | 1.2 | dos Santos et al. (2013) | ash (%) | 3.61 | Dowd et al. (1994) |
| $BO_3^{3-}$ (mg $L^{-1}$) | 1.94 | dos Santos et al. (2013) | carbohydrate (%) | 3.42 | Dowd et al. (1994) |
| Ba (mg $L^{-1}$) | 0.54 | Mariano et al. (2009) | Acetaldehyde (g $L^{-1}$) | 0.697 | Dowd et al. (1994) |

| | | | | | |
|---|---|---|---|---|---|
| Cd (mg $L^{-1}$) | 1.06 | Mariano et al. (2009) | Ethanol (g $L^{-1}$) | 3.83 | Dowd et al. (1994) |
| Cr (mg $L^{-1}$) | 0.15 | Mariano et al. (2009) | Propylene Glycol (g $L^{-1}$) | 0.084 | Dowd et al. (1994) |
| Ni (mg $L^{-1}$) | 0.26 | Mariano et al. (2009) | 2,3-butanediols (g $L^{-1}$) | 0.568 | Dowd et al. (1994) |
| Al (mg $L^{-1}$) | 72.5 | Mariano et al. (2009) | glycerol (g $L^{-1}$) | 5.86 | Dowd et al. (1994) |
| $MoO_4^{2-}$ (mg $L^{-1}$) | 0.17 | dos Santos et al. (2013) | Erythritol (g $L^{-1}$) | 0.088 | Dowd et al. (1994) |
| Cu (mg $L^{-1}$) | 0.06 | Mariano et al. (2009) | Arabinitol (g $L^{-1}$) | 0.064 | Dowd et al. (1994) |
| **Physicochemical analysis** | | | | | |
| density (g $mL^{-1}$) | 1 | Mariano et al. (2009) | chiro-inositol (g $L^{-1}$) | 0.114 | Dowd et al. (1994) |
| pH | 4.84 | Dowd et al. (1994) | Sucrose (g $L^{-1}$) | 0.222 | Dowd et al. (1994) |
| OD (mg $L^{-1}$) | 4.3 | Mariano et al. (2009) | acetic acid (g $L^{-1}$) | 1.56 | Dowd et al. (1994) |
| moisture (%) | 89.64 | Dowd et al. (1994) | formic acid (g $L^{-1}$) | 0.582 | Dowd et al. (1994) |
| Eh (mV) | 260 | Mariano et al. (2009) | lactic acid (g $L^{-1}$) | 7.74 | Dowd et al. (1994) |
| conductivity (mS) | 8.52 | (Mariano et al., 2009) | quinic acid (g $L^{-1}$) | 0.508 | Dowd et al. (1994) |

The major organic compounds present in sugarcane vinasse are glycerol, lactic acid, ethanol, and acetic acid, with residue levels of sucrose (Dowd et al., 1994). Vinasse is also characterized as a feedstock rich in phenolic compounds and melanoidins (FitzGibbon et al., 1998). Using NMR and FTIR, Benke et al. (1998) detected levels of cellulose and hemicellulose in vinasse, which are derived from the grinding conditions of sugarcane. Sugarcane vinasse is reported to be a nitrogen-deficient medium, which is most composed as acid-insoluble nitrogen (Parnaudeau et al., 2008).

Vinasse has been mostly used on fertirrigation practices, i.e., utilizing it as a liquid fertilizer for crops, reducing the water input for plant growth (Walter et al.,

2011). Fertirrigation usually has negative effects on soil and ground waters in the long term (Rocha et al., 2007). A few adequate uses for vinasse management have been identified and used in large-scale operations, such as vinasse recycling to fermentation streams (Fadel et al., 2014; Yang et al., 2016), fertirrigation (Christofoletti et al., 2013; Filoso et al., 2015), energy production (Cortez et al., 1992; Walter et al., 2011), and animal feed production (Cortez et al., 1992). Recently, studies on transforming vinasse into a high-value added feedstock have also been performed (Nitayavardhana and Khanal, 2010). Sugarcane and ethanol production in Brazil has been largely criticized due to several ecological factors (Sparovek et al., 2009), and negative environmental impacts, especially to the increase of contaminants in soil and surface water (Jiang et al., 2012). The use of mature technologies, yet not widespread in the sugarcane-to-ethanol industry, could help attenuate environmental concerns. Oxidation and chemical processes, anaerobic digestion, and microbial fermentation have been presented as alternative impactful alternatives to (i) reduce its organic and mineral load, converting it to a feedstock with fewer environmental applications when applied as fertilizer, and (ii) to convert organic matter and nutrients to a nutritious biomass, simultaneously increasing water reclamationotential by plants. This review article describes some of the alternative uses to

diminish the environmental impact by vinasse management practices, as shown in figure 9.1.

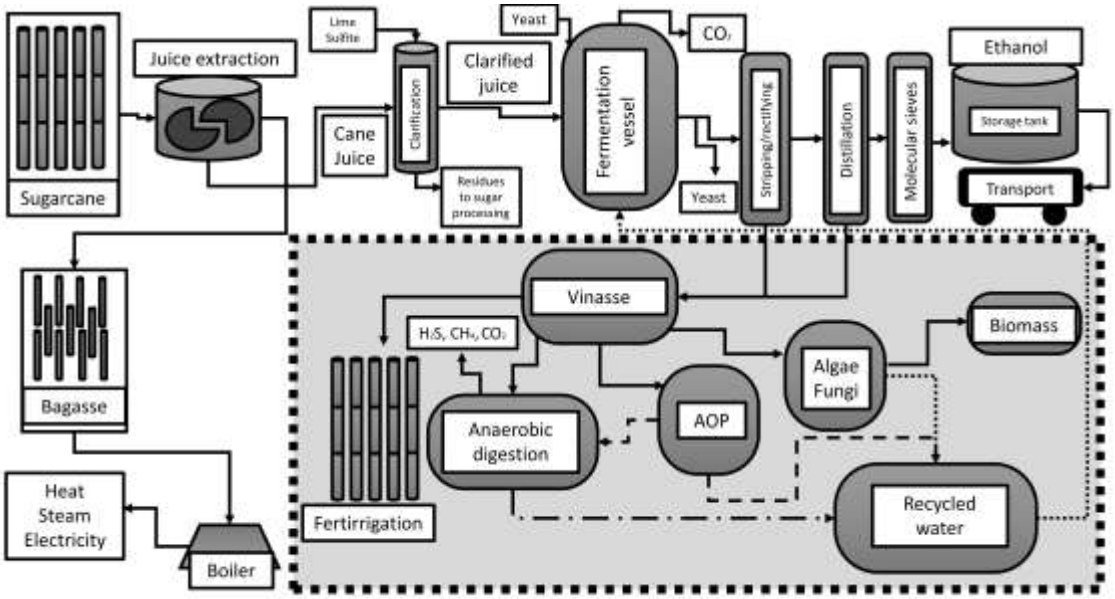


Figure A2.1.: Simplified sugarcane-to-ethanol process with potential modifications in vinasse management

**Fertirrigation practices with vinasse**

The utilization of vinasse in fertirrigation practices started in the 1950's (Valsechi and Gomes, 1954), and by the 1980's, it was a common practice for sugarcane refineries to utilize the liquid residual as fertilizer (Walter et al., 2011). The concept behind fertirrigation consists on a sum of irrigation to sugarcane fields, by the percolation of vinasse liquid to the soil, with the simultaneous fertilization, transferring its nutrients to the plants (Christofoletti et al., 2013). Besides decreasing the costs involved with chemical fertilizers (Jiang et al., 2012), completely supplying phosphorus (Moran-Salazar et al., 2016) and being of low capital cost, vinasse utilization in fertirrigation practices could be considered of certain level of environmental concern (Sparovek et al., 2009). Fertirrigation

practices have been linked with increase in eutrophication of waterbodies and the formation of dead aquatic bodies in Brazil and in other countries (Eykelbosh et al., 2015). The correct application of fertirrigation has proven not to impact the physical, chemical, and biological properties of the soil to which vinasse is applied (Christofoletti et al., 2013), such as, and levels up to 300 $m^3$ vinasse $ha^{-1}$ with potassium levels of 3 to 4 kg $m^{-3}$ do not impact negatively the soil (Penatti et al., 1988). However, conditions with incrase in crop losses, soil pH change, increase in phytotoxicity, and release of sulfurous odors are not uncommon (Christofoletti et al., 2013).

Sugarcane crops occupy nearly 3 million hectares in the Brazilian state of São Paulo (Có Júnior et al., 2008). Current production of ethanol could supplement up to 80% of sugarcane plantation by fertirrigation (Christofoletti et al., 2013). A study conducted at the Pirapama basin river, home to three ethanol plants in Brazil, producing over 500,000 L of ethanol per day during the peak season, has estimated biochemical oxygen demand disposal rates of 226,335 kg on a daily basis, correspondent to a city of 4.2 million people (Alcoforado de Moraes et al., 2009). The high toxicity potential of vinasse being utilized in fertirrigation practices, may lead to hydrologic, agronomic, and social problems. Since there are no pollution charges applied to sugarcane farmers and ethanol producers, fertirrigation still stands as the predominantly application of vinasse.

**Vinasse as a feedstock for biological treatment**

Vinasse typically has a moisture content of about 93%, and the present organic solids and minerals, such as potassium, calcium, and magnesium (Christofoletti

et al., 2013), may provide a rich culture medium for biological cultivation. Detoxification from soils contaminated by excessive vinasse utilization has been evaluated (Abioye, 2011) , indicating that the organic compounds present in the effluent may serve as nutrient sources for microorganisms, despite the low concentration of nitrogen and phosphorus. Prata et al. (2001) also indicated that the readily available carbon sources in vinasse, such as glycerol, accelerated the degradation of the herbicide ametryn in vinasse-contaminated soils.

*In-situ* biological treatment of vinasse could be a potentially solution. Among different fungi that can utilize vinasse as a substrate for growth, *Rhizopus oligosporus* was grown on a vinasse-rich medium (75% v/v) using an airlift bioreactor (Nitayavardhana et al., 2013). The 2.5-L reactor used for the study had supplementation of nitrogen and phosphorus, and aeration rates ranging from 0.5 to 2.0 vvm, achieving a maximum biomass accumulation of 8.04 g of increase compared to the initial mycelium inoculated. Nitayavardhana et al. (2013) reported a decrease in 80% of COD, and observed that the fungal biomass achieved a high accumulation of protein (around 50%), which could be redirected to livestock production, especially since *R. oligosporus* cultivations usually yield amino acid profiles comparable to those of soybean meal (Lim and Akiyama, 1992), being only deficient in methionine and phenylalanine. *R. oligosporus* is a commonly used starter culture for Indonesian tempeh production, and are known to fully utilize carbon sources rich in sucrose, as the one in vinasse, to grow (Egounlety and Aworh, 2003). Fungal treatment of with

white-rot *Trametes versicolor* was performed in order to evaluate the fungus potential to produce laccase and decrease the concentration of phenol and chromophoric compounds in vinasse (España-Gamboa et al., 2015; España-Gamboa et al., 2017). Achieving 60% removal of COD, and over 80% of total phenol, with a decrease in almost 20% in color, *T. versicolor* has proven to be an excellent laccase-producing microorganisms, achieving production of 1630 laccase units per liter of medium (España-Gamboa et al., 2015). Since the presence of melanoidins, phenols and polyphenols have been described to potentially have negative effects on crop productions (Constabel and Ryan, 1998), bioprocessing technologies, such as the study using *T. versicolor*, can decrease the harmful effects of applying vinasse in fertirrigation.

Utilizing microorganisms to treat vinasse does not only serve for the purpose of reducing COD, and toxic compounds. The utilization of fungi cultivated in vinasse potentially can bring additional revenue to sugarcane-based ethanol plants by providing feed and feed supplements to livestock production. Nair and Taherzadeh (2016) cultivated *Neurospora intermedia* and *Aspergillus oryzae* in vinasse, and observed that an integration to a medium-sized facility, producing 100,000 $m^3$ of ethanol a year, could reach up to 250,000 tons of protein-rich (45 weight %) dry fungal biomass per year. *N. intermedia* and *A. oryzae* are characterized as generally regarded as safe (GRAS) materials (Ferreira et al., 2015; Todokoro et al., 2015), and have been traditionally used in the preparation of traditional dishes in Southeast and East Asia, being recently used in starch-

based ethanol waste streams (Ferreira et al., 2015). Heterotrophic algae have also been reported as potential microorganisms to utilize the nutrients in vinasse to grow. The dark color characteristic to vinasse may be comparable to other dark effluents, such as municipal leachate, and may significantly hinder photoautotrophic growth of algae (Reis et al., 2014b). Therefore, heterotrophic growth may be the most appropriate growth mode for algae in vinasse. An example with green algae *Desmodesmus* sp. indicated slight elevation of pH, low oxygen and low carbon dioxide removal, with a decrease of 52.1% in nitrogen and 36.2% of COD. *Desmodesmus* also achieved high yield of COD to biomass in the first hour of growth (0.5 g $g^{-1}$) and specific growth rate of 0.15 $h^{-1}$. *Scenedesmus sp*. was cultivated on a Guillard-modified medium supplemented with 40% of vinasse, and has been reported as able to grow in rates comparable to the control experiments (Ramirez et al., 2014).

Several opportunities used in starch-based ethanol research could be utilized with vinasse as bioprocess medium, such as those producing ethanol, malic acid, butanol, and many other commodity chemicals (Reis et al., 2017). The production of secondary ethanol using *N. intermedia* and *A. oryzae* on vinasse, for instance, would potentially provide extra 12.6% ethanol produced annually (Nair and Taherzadeh, 2016). The direct use of vinasse as feed material, in a similar fashion as the conditions applied to the U.S. dry-grind corn-to-ethanol industry, may not be suitable with the current composition of vinasse, especially due to the low protein content, and surplus of sulfur. An opportunity is to utilize

protein-rich biomass grown in vinasse as animal feed, which may represent a significant change in the utilization of downstream processing of sugarcane-to-ethanol products.

### Addition of value to vinasse via chemical and advanced oxidation processes

Advanced oxidation process (AOP) methods have been extensively used in wastewater treatment facilities, which principles lies behind the high reactivity of HO• radicals, driving oxidation processes to all sorts of recalcitrant pollutants (Andreozzi et al., 1999). Utilizing AOP to treat vinasse is an opportunity to recycle the water back to the fermentation process, decreasing operational costs and environmental footprint of sugarcane plants. The effectiveness of ozone-based AOP ($O_3$, $O_3$/UV and $O_3$/UV/$H_2O_2$) has been tested on a vinasse-like effluent, achieving a fast kinetic degradation profile ($k = 6.5 \times 10^{-3}$ $min^{-1}$) with $O_3$/UV/$H_2O_2$ and being reported as an economical process (1.31 € $m^{-3}$ $g^{-1}$ of TOC mineralized under optimized conditions) (Lucas et al., 2010). The process behind recovering high-value phenolic compounds in vinasse, present at a concentration within the region of 600 mg $L^{-1}$, is likely to be economically not feasible (Santos et al., 2003).

The combination of AOP with other forms of value addition to vinasse is also a research and commercial opportunity. A study conducted by Siles et al. (2011) evaluated the serialization of short-retention time ozonation, which was responsible to a decrease in over 50% of phenols, with anaerobic digestion. Pre-treated vinasse had anaerobic degradability of around 80% of the total COD, with

enhanced methane yield coefficients and methane production rates enhanced by 13.6% and 41.2% when compared to raw vinasse (Siles et al., 2011). Potential studies with phototrophic microorganisms for production of value-added compounds could be coupled with AOP focused on color removal. Fagier et al. (2016) evaluated $Fe^{2+}$-activated persulfate and peroxymonosulfate oxidation on vinasse, to which an addition of 15 g $L^{-1}$ of coagulant provided over 70% total organic carbon removal, and near 100% of $UV_{254}$ and color removal, which lowers the toxicity levels for phototrophic microorganisms. AOP consist on a series of mature and well-understood steps that can help detoxify vinasse to further utilization and value addition, such as production of protein-rich microbial biomass.

### Anaerobic digestion of vinasse: operation, energy generation, and digestate use

Anaerobic digestion (AD) is a common practice in the current dry-grind corn-to-ethanol industry, being responsible to generate energy and degrade complex organic matter present in the corn stillage (Reis et al., 2017). Despite the composition of sugarcane vinasse differing significantly from wastewaters with high carbon load, to which AD processes are ubiquitous and profitable, the possibility of decreasing the negative impacts to vinasse application in soil usage accelerated the research and development of AD in sugarcane vinasse. The first industrial application in Brazil was built in the 1990's and consisted of an up-flow anaerobic sludge blanket (UASB) reactor in São Martinho mill with a capacity of

5000 $m^3$ (Souza et al., 1992). The biogas generated from the UASB reactor was used on the drying process of yeast.

The use of AD in sugarcane vinasse is characterized according the number of steps, process temperature, and reactor design (Rajeshwari et al., 2000). The use of digestate vinasse was used to cultivate the microalgae *Chlorella vulgaris* (Marques et al., 2013; Candido and Lombardi, 2016) and *Neochloris oleoabundans* (Olguín et al., 2015). Initially, research shows that vinasse was highly toxic to *C. vulgaris* at concentrations greater than 4%, reaching allowable concentrations of about 8.6% after treatment (Marques et al., 2013). *C. vulgaris*, a widely used species in accumulation of valuable microbial lipids (Reis et al., 2014b), was able to achieve specific growth rates of 0.76 $day^{-1}$, higher than the control experiments in nutrient sufficient medium (0.53 $day^{-1}$) (Marques et al., 2013). The use of AD can represent a feasible opportunity for making vinasse an appropriate cultivation medium for microbial cultivation, such as in the use of fungi with resilience to unfavorable conditions of growth (Reis et al., 2014a). *N. oleoabundans* was able to grow on supplementation of vinasse up to 8%, with addition of sodium bicarbonate, achieving lipid concentrations up to 38.5%, high $N\text{-}NH_4^+$ removal (85.2%) and high flocculation efficiency (42% after 30 min) (Olguín et al., 2015).

Due to the high sulfur composition of vinasse, AD produces a sulfur-rich biogas (Barrera et al., 2013), which could be highly corrosive to the burners by the production of $SO_2$. Removal of $SO_x$ from biogas can be accomplished, among

other technologies, through physical processes, such as sulfur-specific membrane filters, or through biological processes (Barrera et al., 2014). Lebrero et al. (2015) evaluated biotrickling filters (BTF) and algal-bacterial photobioreactors (PBR) as alternative to membrane removal. Both BTF and PBR constitute removal efficiencies greater than 98%, with elimination capacities as high as 26 g S-$H_2S$ $m^{-3}$ $h^{-1}$. BTF showed impressive robustness as it was completed revived after a 15 day-shut down, and it was able to utilize the nutrients from vinasse, with exogenous nitrate addition (Lebrero et al., 2015). Lebrero et al. (2015) also reported that PBR supported $CO_2$ removal of 23 ± 11.8%, increasing to 62% at pH of 8.1, with an overall fixation rate of 285 mg $CO_2$ $L^{-1}$ $d^{-1}$, thus it could be coupled with a BTF to upgrade AD-generated biogas, removing inert gases and sulfur-rich compounds. Overall, AD of vinasse could generate approximately 4.5 MW yearly, which would correspond to over 14.5 million $m^3$ of biogas, with concentrations of 60% $CH_4$, replacing up to 12% of the bagasse from burning to combined heat and power operations (Moraes et al., 2014). AD in a large-scale sugarcane ethanol plant has a potential of supplying electricity to a city of 130,000 inhabitants or a replacement up to 40% of the annual diesel supply in the agricultural energy requirements of a sugarcane biorefinery (Moraes et al., 2014). Therefore, optimization and widespread use of AD in sugarcane ethanol plants still remain as opportunities.

**Conclusion**

While for many waste sources, a broad literature may be available (e.g. municipal wastewater), for others, references are scarcer and many opportunities

are still under-performed – such is the case for sugarcane vinasse. The use of traditional practices, such as fertirrigation, may cause environmental issues in water and soil quality, and the use of more robust approaches must be a practical solution for vinasse management. Fertirrigation is often a practice that provides a false impression of solving the problem of vinasse disposal. The use of AD is an underperformed process in sugarcane plants, and could significantly increase the energy output, while reducing the amount of water used within a plant. AOP can help decrease the toxicity of vinasse, and can be used as a pretreatment for microbial growth, which could generate high throughput of value-added chemicals. Treatment of vinasse prior to recycling it as fertilizer and irrigation water would potentially lower the environmental impact of applying a nutrient-rich suspension to soils. Thus, vinasse management is an issue in ethanol plants in Brazil, home to the second largest ethanol production in the world, which represents a realm of hidden opportunities for mature technologies.